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On the function of the Ruptured Ovarian Follicle of the Domestic Fowl*

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The relatively few accounts dealing directly with the ruptured avian follicle are limited largely to descriptions of histological changes incident to its regression and to discussions, notably lacking in experimental support, of presumed homology or lack of homology with the corpus luteum of the mammal.[1-4] In any case, previous investigators and writers have been unable to assign any demonstrable physiological function to the ruptured follicle of birds.

In this report we wish to present evidence for the conclusion that the ruptured follicle of the hen participates decisively in control of time of lay of the egg normally arising from ovulation of its previously contained ovum or yolk. Some evidence suggests that the ruptured follicle also participates in regulation of the interval between successive ovulations. At this time, however, we shall consider only those relations demonstrating the dependence of time of lay of the oviducal egg on its cor-

* This investigation was carried out under grant of funds provided by the Bankhead-Jones Act.

1 Pearl, R., and Boring, A., *Am. J. Anat.*, 1918, **23**, 1.

2 Hett, J., *Verh. Anat. Ges.* (Jena), 1922, **31**, 153.

3 Novak, J., and Duschak, F., *Zeit. Ges. Anat.* (Munchen), Abteil I, 1923, **69**, 483.

4 Fell, H. B., *Quart. J. Micr. Sci.*, 1925, **69**, 591.

responding ruptured follicle, leaving aside also all questions of the mechanism responsible for this specific function.

Material and Methods. All hens used in this study were selected from an extensive stock kept in individual laying cages. Most of the birds were from standard breeds, but some cross-bred hens were included. The hens were subjected to a constant 14-hour light-day and were given unlimited access to feed and water. Lay of eggs was recorded hourly from 8:00 a.m. through 4:00 p.m. and again at 8:00 p.m. for a sufficient time before operation to establish the individual hen's schedule of lay. Lay of eggs following operative procedures was recorded similarly as well as at hourly intervals after 4:00 p.m. whenever called for by possibly modified schedules of lay.

The experiments reported here consisted in removing maturing or ruptured follicles (or both simultaneously) and determining the effect on time of lay of eggs in oviducts at the time of operation. It was therefore essential to know accurately the time relations underlying ovulation and lay in individual hens. All hens operated on were known, on the basis of records of lay, to be highly regular in laying schedule and to miss lay on but a single day between clutches (*i.e.,* eggs laid on successive days). Moreover, the oviducal egg was always accounted for at the time of operation, and by reference to hour of lay of the preceding egg, all possibility of this egg having been ovulated at an unusual hour was eliminated.

The ovulated ovum of the hen remains in the oviduct from 24 to 30 hours, the exact time varying with the number of eggs in the clutch and the place of a given egg in the series. Ovulation occurs about 0.5 hour following a previous lay except following lay of the last egg of the clutch, which takes place during afternoon hours generally.[5] The next ovulation, initiating a new clutch sequence in such hens as were used in our tests, occurs during the early morning hours (4:00-8:00 a.m.) of the day following clutch termination, *i.e.,* some 16 to 20 hours following lay of the last egg of the preceding clutch. On the basis of these known relations and the individual hen's record of lay it is possible to ascertain, with a relatively high degree of accuracy, the day and hour of origin of any ruptured follicle borne by the ovary, age of an oviducal egg, and hour of at least the next-due ovulation, which of course is the same as the hour of expected origin of the next-due ruptured follicle.

Like the maturing follicles, the ruptured follicles are pendant to the ovary, and excision of either type is relatively simple. Immediately following ovulation the ruptured follicle weighs about 0.4 to 0.5 g. It loses weight rapidly and decreases correspondingly in size, making identification of any one of at least the 3 largest ruptured follicles practically certain on direct inspection. As a final check, however, each excised follicle was weighed on removal for comparison with expected weight based on recentness of its origin.

Identification of maturing follicles was carried out similarly. Only the largest follicle in the oncoming series was removed in tests reported here. Upon removal, this follicle was weighed and its weight referred to expected weight at maturity, due account being taken of time at which the follicle was removed. The results of Table I do not include any follicle, maturing or ruptured, of questionable place in its appropriate series.

All surgical operations were carried out under Nembutal anesthesia. Operative details were essentially similar to those previously described by Phillips and Warren.[5] The pedicle of the maturing or ruptured follicle was clamped with a hemostat before the desired structure was removed. Whenever possible, the severed stalk was ligated, but bleeding in any case was never excessive and was easily controlled. Twenty to 30 minutes were usually required for the complete operation. Moderately aseptic conditions were maintained, and in most cases sulfathiazole was used sparingly in local wound areas. With very few exceptions the birds operated on exhibited practically no subsequent effects except those immediately involving the ovary or the oviducal egg.

Results. The operated birds of the first

[5] Phillips, R. E., and Warren, D. C., *J. Exp. Zool.,* 1937, **76**, 117.

TABLE I.
The Effect of Removing Ruptured and Maturing Follicles on Retention of the Egg in the Oviduct at Time of Operation.

Group	Follicle removed	Age of oviducal egg, hr	Birds		Duration of delay in laying, range
			Operated on, No.	Retaining eggs, No.	
I	R_1	0.5-26	22	17	9 hr to 3 days
II	M_1	0.5-24	30	2	3 and 5 hr
III	R_1 and M_1	2.5-25	15	14	1 to 7 days
IV	R_2	1.0-21	18	4	2 to 5 hr

R_1—The most recently ruptured follicle.
R_2—The second ruptured follicle in order of recency of origin.
M_1—The oldest maturing follicle, next due to ovulate.

experiments constituted Group I and II of Table I. The most recently ruptured follicle was removed from hens in Group I, the oldest maturing follicle from hens of Group II. Of the 17 hens holding their eggs following removal of the most recently ruptured follicle, only 1 held for as little as 9 hours; 8 hens held their eggs for 1 day, 4 for 2 days, and 4 for 3 days. In sharp contrast with these results, all except 2 hens of Group II laid at the expected hour. The 2 hens holding their eggs for 3 and 5 hours appear to belong to a definite category which will be discussed later.

Both the most recently ruptured follicle and the most mature of the maturing follicles were removed from the hens of Group III. Of the 15 operated hens, 14 held their eggs over the following intervals: 5 for 1 day, 3 for 2 days, 2 for 3 and 2 for 5 days, and 1 each for 6 and 7 days.

As a final check on the foregoing results the second most recently ovulated follicle was removed from 18 birds, the most recently ruptured follicle being left intact (Group IV of Table I). Fourteen hens laid at the expected hour, and 4 held their eggs for periods not exceeding 5 hours. These are discussed in connection with the 2 similarly holding hens of Group II.

Not shown in Table I is an additional group of 17 hens on which sham operations were performed, the follicles being handled but not removed, or the birds being carried through other phases of complete operation. Of these, 16 laid at the expected hour and one hen held its egg for a period of one day, possibly as the result of follicular injury although this cannot be said with certainty.

Discussion. Follicular regression was brought about in many of the operated birds, with resultant interruption of ovulation for 1 or more days. In view of the normally close relation between ovulation and lay (excepting always the terminal egg of the clutch), it might therefore be argued that we have prevented lay not by removing the organ directly controlling this process, but rather by causing follicular regression and thus preventing ovulation. In the hens of Group II, however, all possibility of ovulation near the hour of expected lay was eliminated by excision of the ovulable follicle with minor effect in only two of 30 operated birds. Furthermore, only similar minor delays occurred in any of the hens of Group IV, although 17 of the 18 hens failed to ovulate in conjunction with lay. It may be remarked finally that the terminal egg of the clutch of the normal intact hen is laid 16 to 20 hours before the next ensuing ovulation, and is not, therefore, immediately dependent upon ovulation for lay. In view of the evidence from these several lines, we conclude that normally timed lay depends mainly upon some property of the ruptured follicle from which the oviducal egg has taken its origin.

Although the last-ruptured follicle appears to be the dominant factor in control of lay of the oviducal egg, the maturing follicle is not without secondary or substitutional function, within limits. Of the 5 hens in Group I which laid normally, 3 did so in conjunction with normally due ovulation. The higher percentage of birds holding their eggs following excision of both the maturing and ruptured follicles (Group III) is obtained in association with elimination of all possibility of next due ovulation. These results suggest, when

taken together, that the follicle ovulating about the time of lay exhibits some of the properties of the ruptured follicle.

It has been shown that the terminal egg of a clutch remains in the oviduct for an appreciably longer time than do mid-clutch eggs.[6] The 2 birds of Group II and the 4 of Group IV, in which lay was delayed only a few hours, were entirely dependent upon the most recently ruptured follicle for lay, next-due ovulation having been precluded by operation or regression. The postponement in time of lay by only a few hours is thus approximately comparable with the behavior of the terminal egg of a clutch, which egg also is laid in the absence of closely ensuing ovulation.

It would be premature to speculate at this time upon the mechanism underlying the action of the ruptured follicle in determining the hour of lay of its ovulated yolk. We believe, however, that the results presented here provide adequate proof that the avian ruptured follicle is not an organ without function, but that this structure, like its mammalian homologue, exerts an influence on the oviducal history of the ovum it once contained. Since the oviducal histories of ova of the two classes of vertebrates are so dissimilar, it will be interesting to observe what parallels and divergences exist in physiological behavior of the two organs.

Summary. Removal of the ruptured ovarian follicle or of this follicle together with the oldest maturing follicle of the hen at the time when the egg taking its origin from the ruptured follicle was in the oviduct almost invariably resulted in the holding of the egg beyond the expected time of lay. Most eggs were held from 1 to 7 days. Removal of other parts of the ovary at comparable times, without simultaneously removing the most recently ruptured follicle, practically never resulted in comparable holding of the oviducal egg. The ruptured follicle of the hen is believed therefore to be an important factor in determining the time of lay of the egg formed from its previously contained ovum.

[6] Heywang, B. W., *Poultry Sci.*, 1938, **17**, 240.

14601

Treatment of Experimental Renal Obstruction from Sulfadiazine. I. "Forcing of Fluids" and Alkalinization.*

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The value of massive alkalinization in the prevention of renal complications from intratubular precipitation of sulfadiazine and its acetylated product has been established clinically[1,2,3,4] as well as in the animal experiment.[5,6] The application of this procedure in combination with the "forcing of fluids" has also been suggested in fully developed renal obstruction and anuria occurring during the course of sulfadiazine therapy,[7] but its usefulness and advisability lacks experimental foundation.

* This investigation has been aided by a grant from the Josiah Macy, Jr., Foundation.

[1] Gilligan, D. R., Garb, S., and Plummer, N., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 248; Gilligan, D. R., Garb, S., Wheeler, C., and Plummer, N., *J. A. M. A.*, 1943, **122**, 1160.

[2] Barnes, R. W., and Kawaichi, G. K., *J. Urol.*, 1943, **49**, 324.

[3] Jensen, O. J., Jr., and Fox, C. L., Jr., *J. Urol.*, 1943, **49**, 334.

[4] Fox, C. L., Jr., Jensen, O. J., Jr., and Mudge, G. H., *J. A. M. A.*, 1943, **121**, 1147.

[5] Lehr, D., *Bull. New York M. College, Flower and Fifth Ave. Hosps.*, 1943, **6**, 70.

[6] Jensen, O. J., Jr., *Am. J. Med. Sci.*, 1943, **206**, 746.

[7] Lowell, F. C., *M. Clin. North America*, Sept., 1943, p. 1247.

It was the purpose of this investigation to evaluate the merits of alkalinization in conjunction with the "forcing" of water, after the production of a renal block by intratubular precipitation of sulfadiazine under the standard conditions of the animal experiment. Included in this study for the purpose of comparison was an evaluation of the effects of "forcing" either water alone, or solutions containing an acidifying salt or a salt mixture.

Procedure. Young albino rats from our own standard colony, weighing 150 to 200 g, were used in all experiments. The animals were kept on a standard diet (Rat Food, Rockland Farms) and had free access to drinking water.

Drug precipitation in the kidney tubules—in accordance with a finding reported originally for sulfathiazole[8]—was produced by intraperitoneal injection of a single fatal dose of sodium sulfadiazine† (1.5 g per kg body weight). If left untreated, the animals without exception develop, within a few hours, pronounced and long lasting renal obstruction from intratubular precipitate of sulfadiazine, and 70 to 80% succumb to this complication after 2 to 3 days. (This observation is based on chemical findings in blood and urine, and post mortem examination of 50 animals.) A detailed analysis of the mechanism causing death has been presented elsewhere.[9]

Treatment consisted in stomach tube feedings of fixed amounts of fluid (water or salt solutions), twice daily, starting shortly before the sulfadiazine injection and continuing for at least one or several more days. All salt solutions were tolerated by control animals for several days with little ill effect and with no significant change of the blood pH.

Throughout the experimental period, volume, specific gravity, pH (measured with a Cambridge Electron-Ray pH meter), and sulfadiazine concentration of the urine were recorded daily, and determinations of nonprotein nitrogen, drug level and pH were carried out in the blood at various intervals. All surviving rats were killed by exsanguination in ether narcosis, usually 8 to 10 days after completion of each particular experiment. Thorough post mortem examinations were performed on these animals and also on those succumbing during the experiment. Special attention was given to the appearance of the urinary tract. The most important organs of representative animals were fixed in formaldehyde for histological study.

Results. The results of different experiments although similar in their main features, varied considerably in accordance with the duration of the treatment. Undue prolongation of therapy usually increased the mortality. For the purpose of this report it seemed sufficient to present some significant findings of one typical experiment, performed with 60 closely bred, 3 months old, female rats. The data are summarized in Table I.

This particular experiment was run in 2 parallel sets. Subgroups of 5 rats each were placed in separate metabolism cages. Chemical studies were done on the second set (animals No. 6-10) only, with all animals which were still alive 48 hours after the injection of sulfadiazine. Therapy (forcing of fluids) was started 3 hours before the sulfadiazine injection and continued for one more day (4 stomach tube feedings in all).

The most striking result apparent from Table I is the excellent therapeutic success achieved with solutions of sodium chloride and of salt mixture (Groups B and E). They made possible the survival and complete recovery of all rats from an otherwise fatal sulfadiazine intoxication, whereas no benefits were derived from the "forcing" of water alone (Group A). Sodium bicarbonate (Group D), on the other hand, very definitely shortened the time of survival (maximum 48 hr), and ammonium chloride (Group C), in addition to a further reduction of the life span (24 hr), also increased the mortality to 100%.

From the chemical data obtained in this particular test, the consistent impairment of renal function is obvious in groups A, B, E, and F (uniformly pronounced elevation of the nonprotein nitrogen values in the blood); the sulfadiazine blood levels, however, taken at

8 Lehr, D., Antopol, W., and Churg, J., *Science*, 1940, **92**, 434.

† The sodium sulfadiazine used in this study was supplied by the Schering Corporation, Bloomfield, N.J.

9 Lehr, D., and Antopol, W., *Urol. and Cutan. Rev.*, 1941, **45**, 545.

TABLE I.

Fatal Intoxication of Albino Rats with Sulfadiazine.

(Single intraperitoneal injection of 1.5 g of sodium sulfadiazine per kg body weight in 5% aqueous solution.)

Forcing of fluids (3 ml per 100 g body wt) by stomach tube twice daily	Rat No.	Died within days			% dead	48 hr after injection of SD			
						Blood		Urine per rat (mean value)	
		1	2	3		N.P.N. mg%	SD mg%	SD mg	Vol. ml
(A) Water	1								
	2		X						
	3		X						
	4		X						
	5		X		80				
	6			X		215	116		
	7					205	124		
	8		X					42	19
	9		X						
	10		X						
(B) Aqueous sol. of NaCl, 3.33%	1								
	2								
	3								
	4								
	5				0				
	6					227	73		
	7					205	71		
	8					199	94	65	38
	9					215	87		
	10					199	91		
(C) Aqueous sol. of NH_4Cl, 1.67%	1	X							
	2	X							
	3	X							
	4	X							
	5		X		100				
	6	X							
	7	X							
	8	X						7	5
	9	X						(24 hr values)	
	10	X							
(D) Aqueous sol. of $NaHCO_3$, 3.33%	1		X						
	2								
	3								
	4		X						
	5		X		80				
	6		X						
	7		X						
	8		X					70	21
	9		X						
	10		X						
(E) Aqueous sol. containing *both* NH_4Cl, 1.67%, $NaHCO_3$, 3.33%	1								
	2								
	3								
	4								
	5				0				
	6					179	54		
	7					199	98		
	8					222	75	107	51
	9					160	51		
	10					222	76		

TABLE I. (Continued)

Forcing of fluids (3 ml per 100 g body wt) by stomach tube twice daily	Rat No.	Died within days 1	2	3	% dead	48 hr after injection of SD: Blood N.P.N. mg%	Blood SD mg%	Urine per rat (mean value) SD mg	Urine per rat (mean value) Vol. ml
(F)	1								
Control	2								
No treatment of	3		X						
SD intoxication	4		X						
	5			X	80				
	6			X		207	118		
	7		X			216	135		
	8			X		219	131	38	16
	9			X		227	128		
	10		X						

the same time, indicate a much more rapid clearance of the drug in the sodium chloride and salt mixture groups (B and E). The observation is confirmed by the high amounts of sulfadiazine excreted in the urine of these two latter groups, and finds its explanation in the successful abolition of the renal block in the surviving animals. After 4 days most of the survivors showed only traces of sulfadiazine in the blood and the nonprotein nitrogen levels had fallen to almost normal. The disappearance of kidney obstruction can also be inferred from the large volumes of urine. Massive alkalinization, on the other hand, although it increases the solubility of sulfadiazine and thus permits the initial elimination of large amounts of drug (high urinary concentrations), does not succeed in keeping the urinary pathways sufficiently open to prevent the fatal accumulation of alkali in the body.

In experiments, otherwise identical, blood determinations were performed 20 hours after sulfadiazine injection, including estimation of the pH. This shorter interval permitted the inclusion of the sodium bicarbonate and ammonium chloride animals in the blood analysis. The determinations revealed very high and strikingly uniform nonprotein nitrogen and sulfadiazine values (N.P.N., 170-200 mg %; sulfadiazine "total," around 150 mg %) in all groups, indicating that no animal escaped initial renal obstruction. However, there were very significant differences in the serum pH values obtained from heart blood. They indicated the presence of a severe uncompensated acidosis (pH 6.80 to 7.15) in the ammonium chloride group, whereas those from the bicarbonate animals pointed to the development of an uncompensated alkalosis (pH 7.67 to 7.75). The pH values of all other groups remained within the normal range (pH 7.35 to 7.60), with the sodium chloride animals at the lower limit of the normal and somewhat exceeding it.

The remarkable therapeutic effect of *simultaneous* administration of ammonium chloride and sodium bicarbonate (Group E) in exactly the same concentrations, which, when given separately, had such deleterious effects (Groups C and D), demonstrates that the mixture of an acidifying and an alkalinizing salt apparently prevents dangerous changes in the pH of the blood, while the increased crystalloid concentration enhances the diuretic effect of the solution, possibly by inhibiting the reabsorption of water in the renal tubules. This contention would also explain the therapeutic action of a hypertonic sodium chloride solution (Group B), despite the fact that sodium chloride is a slightly acidifying agent.

Since all animals had free access to water, the thirst stimulus caused by the administration of hypertonic salt solutions resulted in a considerably increased water intake in the saline and salt mixture group, particularly after the establishment of a pronounced diuresis. This observation makes the administration of hypertonic salt solutions comparable to the "forcing" or much larger volumes of isotonic solutions. Preliminary re-

sults from experiments with physiological saline, now under way, substantiate this contention.

Summary. A simple method is presented which permits accurate comparative studies on the therapy of experimental renal obstruction from sulfadiazine. Under the conditions of this method, the results of experiments completed up to now, suggest that in fully developed renal obstruction from sulfadiazine, the generally recommended therapeutic measures—"forcing of fluids" with or without massive alkalinization—can be useless or even harmful, whereas, under identical conditions, solutions of neutral salts or of moderately alkalinizing salt mixtures are able to reopen the urinary pathways quickly and thus to save life.

The author wishes to express his appreciation to Miss Helen Salzberg and to Miss Ruth Levi for valuable technical assistance.

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Effect of Epinephrine on the Synthesis of Acetylcholine.*

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Stimulation of the sympathetic nervous system or administration of epinephrine is often followed by manifestations similar to those that follow administration of acetylcholine. Epinephrine was found to inhibit choline esterase *in vitro*.[1,2] In the following it was ascertained whether the presence of epinephrine modifies the synthesis of acetylcholine *in vitro*.

Method. The synthesis of acetylcholine was studied following the method of Quastel, Tennenbaum, and Wheatley[3] with minor modifications.[4] Hundred mg samples of homogenized fresh frog brains were used as a source of the enzyme, while human serum and human spinal fluid (1 cc) and the frog brain itself supplied the substrate. Three mg physostigmine salicylate, to prevent the enzymatic hydrolysis of the acetylcholine, 4.8 mg glucose, to supply an excess of the precursor of the acetyl-radical and 2 cc Ringer's solution were added to each mixture. Varying amounts of epinephrine (0.03 μg to 30 μg) were added to the mixtures, and the pH of the mixtures was adjusted to 7.4. Identical mixtures without epinephrine served as controls. The mixtures were shaken and incubated aerobically for 4 hours at 37°C. After shaking the mixtures were diluted to 10 cc with Ringer's solution and centrifuged. The acetylcholine content was assayed biologically using the sensitized rectus abdominis muscle of frog; the amount of acetylcholine synthesized was computed by subtracting the acetylcholine content of identical, non-incubated mixtures from the acetylcholine content of incubated mixtures. The free acetylcholine synthesized was determined from the acetylcholine content of the supernatant fluid; the total acetylcholine† synthesized, from a solution obtained by resuspending the precipitate in the supernatant fluid, boiling for 2 minutes at pH 6.8, centrifuging and cooling. Since epinephrine in the concentrations used did not induce muscle contraction by itself, and since it did not modify the effect of acetylcholine in inducing muscle contraction, the use of the rectus abdominis muscle was a valid tool for determin-

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1 Ammon, R., *Pflüger's Arch.*, 1933-34, **233**, 486.

2 Waelsch, H., and Rackow, H., *Science*, 1942, **96**, 386.

3 Quastel, J. H., Tennenbaum, M., and Wheatley, A. H. M., *Bioch. J.*, 1936, **30**, 1668.

4 Torda, C., and Wolff, H. G., *J. Clin. Invest.*, 1944 in press.

† Total acetylcholine is the sum of free and bound intracellular and extracellular acetylcholine.

TABLE I.
Amount of Acetylcholine Synthesized in the Control Mixtures.

Mixtures containing frog brain (100 mg), Ringer's sol. (2 cc), physostigmine salicylate (3 mg), glucose (4.8 mg) and 1 cc of	Acetylcholine synthesized in μg/100 mg of frog brain	
	Free acetylcholine	Total acetylcholine
Spinal fluid	2.11 ± 0.053	3.16 ± 0.049
Serum	1.45 ± 0.011	2.08 ± 0.029
Ringer's solution	0.41 ± 0.013	1.02 ± 0.033

TABLE II.
Effect of Epinephrine on the Synthesis of Acetylcholine *in vitro*.

Final conc. of epinephrine	Amount of acetylcholine synthesized in % of control*					
	Free acetylcholine			Total acetylcholine		
	Spinal fluid	Serum	Ringer's sol.	Spinal fluid	Serum	Ringer's sol.
0	100	100	100	100	100	100
1.10^{-8}	145	139	151	139	138	147
1.10^{-7}	149	153	160	152	169	172
1.10^{-6}	163	168	178	180	190	104
1.10^{-5}	206	230	115	210	225	250

*Each value represents the average of 8 separate experiments. The S.E. of the mean for each value was less than ±10%.

ing the acetylcholine content of the mixtures.

Results. The amounts of acetylcholine synthesized in the controls are summarized in Table I, those synthesized in the presence of epinephrine in Table II. Epinephrine in concentrations from 1.10^{-8} to 1.10^{-5} increased the synthesis of acetylcholine by 40 to 150% respectively above the control. The increase of synthesis of acetylcholine was observed in mixtures containing frog brain alone as substrate and also in those containing an additional amount of serum or spinal fluid.

Discussion. Epinephrine increases the synthesis of acetylcholine *in vitro*. This increase is significant and manifests itself even with low concentrations of epinephrine. An adequate explanation of the mechanism of this potentiation by epinephrine cannot be offered yet. The lowest concentrations used in the above experiments are within the concentrations of epinephrine in the blood.[5]

Assuming that similar increase of the synthesis of acetylcholine may occur in the body, it is possible that the succession of manifestations of stimulation of the sympathetic nervous system by manifestations similar to those that follow administration of acetylcholine is due to both an increase in synthesis of acetylcholine and an inhibition of choline esterase.

Summary. 1. The synthesis of acetylcholine *in vitro* in the presence of epinephrine was investigated. 2. Epinephrine in concentrations used (1.10^{-8} to 1.10^{-5}) increased the synthesis of acetylcholine by 40 to 150% respectively above the control.

[5] Stewart, G. N., and Rogoff, J. M., *Endocrinology*, 1922, **2**, 127.

14603

Effect of Vitamin B_1 and Cocarboxylase on Synthesis of Acetylcholine.*

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Thiamine chloride and the pyrophosphate ester of thiamine chloride are potent inhibitors of choline esterase.[1,2,3,4] The concentrations required for such inhibition are higher than those found in the serum, but probably do not exceed the concentrations present in some tissues. Yet, thiamine chloride, even in higher concentrations, does not help patients benefited by other inhibitors of choline esterase (myasthenia gravis). The above observations suggested the investigation of the effects of thiamine compounds on the synthesis of acetylcholine.

Method. The synthesis of acetylcholine was studied following the method of Quastel, Tennenbaum, and Wheatley[5] with minor modifications.[6,7] Varying amounts of thiamine chloride (from 2.7 μg to 2.7 mg) and of thiamine pyrophosphate (from 2.8 μg to 2.8 mg) were added to mixtures containing enzyme (100 mg minced frog brain), substrate (1 cc serum or spinal fluid or Ringer's solution), physostigmine salicylate (3 mg) glucose (4.8 mg), and Ringer's solution (2 cc), and the pH of the mixtures was adjusted to 7.4. Identical mixtures without the thiamine compounds served as controls. The mixtures were shaken and incubated aerobically for 4 hours at 37°C. After incubation the free and total acetylcholine synthesized was determined biologically using the sensitized rectus abdominis muscle of frog. Since the thiamine compounds, in the concentrations used, did not induce muscle contraction, and since they did not significantly modify the effect of acetylcholine in inducing muscle contraction, the use of the rectus abdominis muscle was a valid means of determining the acetylcholine content of the mixtures.

Results. The amounts of acetylcholine synthesized in the presence of the thiamine compounds are summarized in Table I. Both thiamine chloride and thiamine pyrophosphate in biological concentrations (3.10^{-6} M) increased the synthesis of acetylcholine slightly (average 10%). Mann and Quastel,[8] using thiamine chloride, found a similar increase. Both thiamine chloride and thiamine pyrophosphate, in higher than biological concentrations, decreased the synthesis of acetylcholine. Thiamine chloride was a more potent depressor than was thiamine pyrophosphate. Changes in the synthesis of acetylcholine (as expressed in percentage) due to the presence of the thiamine compounds were similar in mixtures containing only frog brain, frog brain and serum, and frog brain and spinal fluid.

Discussion. An adequate explanation of the mechanism through which the thiamine compounds increase and depress respectively the synthesis of acetylcholine cannot be offered. It is possible that the slight increase of synthesis is due to the direct effect of cocarboxylase on the carbohydrate metabolism, resulting in a better supply of the acetyl-radical. It is possible that the decrease of synthesis of acetylcholine is due to a direct reaction of large amounts of the thiamine compounds with the enzyme involved in the synthesis of acetylcholine. Such reaction cannot

* This study was aided by a grant from the Josiah Macy, Jr., Foundation.

1 Glick, D., and Antopol, W., *J. Pharm. Exp. Therap.*, 1939, **65**, 389.

2 Sullmann, H., and Birkhauser, H., *Schweiz. Med. Wschr.*, 1939, **69**, 648.

3 Massart, L., and Dufait, R., *Naturwissenschaften*, 1939, **27**, 567.

4 Glick, D., and Antopol, W., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 396.

5 Quastel, J. H., Tennenbaum, M., and Wheatley, A. H. M., *Bioch. J.*, 1936, **30**, 1668.

6 Torda, C., and Wolff, H. G., *J. Clin. Invest.*, 1944, in press.

7 Torda, C., and Wolff, H. G., in press.

8 Mann, J., and Quastel, J. H., *Nature*, 1941, **145**, 856.

TABLE I.
Effect of Thiamine Compounds on Synthesis of Acetylcholine *in vitro*.

Substance	Conc. in mols	No. of exp.	Amounts of acetylcholine synthesized in % of control*: Free acetylcholine — In the presence of frog brain and Spinal fluid	Serum	Ringer's sol.	Total acetylcholine — In the presence of frog brain and Spinal fluid	Serum	Ringer's sol.
(Control) †			100 (1)	100 (2)	100 (3)	100 (4)	100 (5)	100 (6)
Thiamine chloride	3.10^{-6}	8	114	108	117	116	109	120
	3.10^{-5}	8	87	93	90	73	80	70
	3.10^{-4}	8	60	68	71	66	65	55
	3.10^{-3}	8	40	44	38	40	37	36
Thiamine pyrophosphate	2.10^{-6}	4	108	104	110	105	108	111
	2.10^{-5}	4	87	90	89	81	84	90
	2.10^{-4}	4	72	77	72	67	72	68
	2.10^{-3}	4	56	54	56	59	60	55

* The S.E. of the mean for each value was less than ±10%.

† The amounts of acetylcholine synthesized in μg per 100 mg frog brain are: (1) 2.11 ± 0.053, (2) 1.45 ± 0.011, (3) 0.41 ± 0.013, (4) 3.16 ± 0.049, (5) 2.08 ± 0.029, (6) 1.02 ± 0.033.

be excluded since both the thiamine compounds and acetylcholine are quaternary ammonium compounds containing a free hydroxyl group, and since the thiamine compounds also have a higher affinity for choline esterase than does acetylcholine. Concentrations of vitamin B_1 and cocarboxylase inhibiting choline esterase also inhibit the synthesis of acetylcholine. Hence, no significant aid can be expected from these substances for patients with disorders resulting from decreased synthesis of acetylcholine.

Summary. 1. The synthesis of acetylcholine *in vitro* in the presence of thiamine chloride and thiamine pyrophosphate was investigated. 2. Both thiamine chloride and thiamine pyrophosphate, in concentrations of 3.10^{-6} M and 2.10^{-6} M respectively, slightly increased the synthesis of acetylcholine (average 10%). 3. The thiamine compounds in higher concentrations depressed the synthesis of acetylcholine. Thiamine chloride is a more potent depressor than thiamine pyrophosphate.

The thiamine pyrophosphate was kindly supplied by the Merck Research Laboratories, Rahway, N.J.

14604

Effect of Thiamine Compounds on the Striated Muscle.*

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Minz[1] observed that cholinergic nerves liberate not only acetylcholine but also thiamine chloride when stimulated. From this observation von Muralt[2] postulated that vitamin B_1 may have some direct effect on the contraction of striated muscles following the stimulation of motor nerves. Vitamin B_1 increases the effect of acetylcholine on the intestine[3,4,5,6] and circulatory apparatus of

* This study was aided by a grant from the Josiah Macy, Jr., Foundation.

1 Minz, B., *C. R. soc. biol.*, 1938, **127**, 1251.

2 von Muralt, J., *Naturwiss.*, 1939, **27**, 261.

3 Binet, L., and Minz, B., *Arch. int. Physiol.*, 1936, **42**, 281.

cat.[4] This phenomenon was explained by the fact that thiamine chloride has an affinity for choline esterase which is 26 times greater than that of acetylcholine.[7] Acetylcholine and thiamine chloride may have similar effects as both are quaternary ammonium compounds containing a free hydroxyl group. The purpose of the following investigation was to ascertain whether thiamine chloride and related compounds have a direct effect on the striated muscle of frog, and whether they modify the contraction induced by chemical agents such as acetylcholine and potassium.

Experimental. 1. *Direct effect of thiamine compounds on the striated muscle.* The rectus abdominis muscle of frog was excised and suspended in a muscle chamber containing 10 cc of Ringer's solution. The Ringer's solution was changed to one of the thiamine compounds for 3 minutes. The muscle was then washed for 20 minutes with Ringer's solution. This procedure was repeated increasing the concentration of the thiamine compounds. The pH of all solutions used here and in the following experiments was corrected to 7. The height of contraction of the muscle was registered by an isotonic lever on a kymograph.

Very high concentrations of the thiamine compounds were required to start a contraction in 3 minutes (thiamine chloride 4.10^{-2} M (average of 10 exp.), thiamine pyrophosphate 1.10^{-2} M (average of 8 exp.), acetyl-thiamine chloride 6.10^{-2} (average of 8 exp.). The sensitivity of the muscle to the thiamine compounds was not significantly increased by immersion for half an hour in a solution of physostigmine containing 2 mg physostigmine salicylate per 100 cc of Ringer's solution.

II. *Effect of thiamine compounds on effect of acetylcholine in inducing muscle contraction.* The rectus abdominis muscle of frog was prepared as before. The Ringer's solution was changed to an acetylcholine solution (50 μg per 100 cc Ringer's solution) for 2 minutes. The muscle was then washed with Ringer's solution for 10 minutes. This procedure was repeated until 3 successive exposures to the solution of acetylcholine gave similar responses. Afterwards the muscle was washed only for 5 minutes and immersed in one of the solutions of the thiamine compounds for 5 minutes. A series of solutions containing the thiamine compounds in increasing concentrations was used.

The thiamine compounds did not increase the effect of acetylcholine in inducing muscle contraction. Thiamine compounds in higher concentrations depressed the effect of acetylcholine (Table I). A similar depression was observed when the thiamine compounds were added directly to the acetylcholine instead of immersing the muscle in the solutions of the thiamine compounds for 5 minutes.

Thiamine compounds dissolved in a solution of physostigmine had a similar effect upon the sensitivity of the eserinized muscle to acetylcholine as they had on the non-sensitized muscles. (These muscles were contracted with a solution containing 10 μg acetylcholine per 100 cc Ringer's solution.)

III. *Effect of thiamine compounds on contraction of muscle induced by potassium.* The rectus abdominis muscle was treated as above, but a 20 mM solution of potassium chloride in Ringer's solution was used instead of acetylcholine. The height of contraction induced by potassium increased significantly in the presence of very low concentrations of the thiamine compounds, and increased with increasing concentrations (Table I). Thiamine pyrophosphate was the most potent agent.

Discussion. The thiamine compounds in concentrations about 100 times as great as found in serum induce a contraction of the striated muscle. The thiamine compounds used, even in very low concentrations, increased the effect of potassium in inducing muscle contraction. These effects are probably due to the action of thiamine compounds on the metabolism of muscle since thiamine pyrophosphate was the most potent agent.

The thiamine compounds in biological and

[4] Agid, R., Beauvallet, M., and Minz, B., *C. R. soc. biol.*, 1937, **126**, 982.

[5] Abderhalden, E., and Abderhalden, R., *Kli. Wschr.*, 1938, **17**, 1195.

[6] Abderhalden, E., and Abderhalden, R., *Pflüger's Arch.*, 1938, **240**, 647, 746.

[7] Glick, D., and Antopol, W., *J. Pharm. Exp. Therap.*, 1939, **65**, 389.

TABLE I.
Effect of Thiamine Compounds on Contraction of Muscle Induced by Acetylcholine and Potassium.

Conc. of the thiamine compounds in mols	Magnitude of contraction in % of control after immersion in the thiamine compounds for 5 min.*								
	Contraction induced by acetylcholine in muscles immersed in			Contraction induced by acetylcholine in sensitized muscles immersed in			Contraction induced by potassium in muscles immersed in		
	Thiamine chloride	Thiamine pyrophosphate	Acetylthiamine	Thiamine chloride	Thiamine pyrophosphate	Acetylthiamine	Thiamine chloride	Thiamine pyrophosphate	Acetylthiamine
0	100	100	100	100	100	100	100	100	100
3.10^{-7}	96	93	94	94	94	98	134	139	132
3.10^{-6}	93	93	92	96	93	93	149	166	162
3.10^{-5}	86	90	82	87	91	89	170	178	172
3.10^{-4}	78	89	68	76	86	64	180	232	207
3.10^{-3}	45	75	38	41	71	35	199	300	218

* Each value represents the average of 12 separate experiments. The S.E. of the mean for each value was less than $\pm 5\%$.

higher concentrations did not increase the effect of acetylcholine in inducing muscle contraction, while in high concentrations the effect of acetylcholine was depressed. The time necessary to exert this depression is very short. An adequate explanation cannot be offered at the present. It is possible that the presence of the thiamine compounds in relatively large concentrations prevents some of the receptor mechanisms from reacting to the presence of acetylcholine.

The above observations may throw some light on the mechanism of action of the thiamine compounds in regulating the contraction of muscle.

Summary. 1. The effect of thiamine chloride, thiamine pyrophosphate, and acetylthiamine in inducing muscle contraction and the effect of the thiamine compounds on the muscle contraction induced by acetylcholine and potassium were investigated. 2. Thiamine compounds did not induce a contraction of the muscle in concentrations lower than 1.10^{-2} M in a period of 3 minutes. 3. The thiamine compounds did not sensitize the muscle to acetylcholine. In higher concentrations the effect of acetylcholine in inducing muscle contraction was depressed. 4. The thiamine compounds significantly increased the effect of potassium in inducing muscle contraction.

Thiamine pyrophosphate and acetylthiamine was kindly supplied by the Merck Research Laboratories, Rahway, N.J.

14605 P

Embryonic Chick Antigens for Complement Fixation with Viruses of Eastern and Western Equine Encephalomyelitis.*

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The complement-fixation test has been increasingly employed in the identification of virus infections of the central nervous system. With one exception all complement-fixing antigens thus far employed with equine encephalomyelitis have been prepared from animal brain tissue, usually that of the mouse.[1-7] Mohler,[8] using a formolized antigen

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prepared from chick embryos inoculated with the virus of Western equine encephalomyelitis, reported complement fixation with serum from convalescent or immunized horses.

Because of its size a single infected chick embryo contains many times as much virus as an infected mouse brain and in view of the greatly decreased incubation period for maximal production of virus further investigation of this source of antigen was suggested.

Equine encephalomyelitis antigen. A strain of Eastern virus obtained from Dr. A. B. Sabin and the McMillan strain of Western virus received from Dr. J. Casals were employed. The viruses had been maintained at high titer by continued intracerebral passage through mice. They were then subjected to 13 rapid passages in hens' eggs containing 10-day-old embryos by inoculation of 0.1 cc of 1:100 suspension of infected embryonic tissue onto the chorioallantoic membrane. The embryos were removed after 18-24 hours and suspended in physiological salt solution to give a concentration of 20% by weight. The suspension was centrifuged at 2,500 r.p.m. for 15 minutes and the supernatant removed and stored at −70°C. Before use as antigen the virus suspension was thawed and centrifuged at 3,000 r.p.m. for 15 minutes. The clear supernatant was diluted with an equal volume of physiological salt solution. The preparations, still infectious for mice in titers of 10^{-8} with Eastern virus and 10^{-6} with Western virus, were then tested in order to determine their complement-fixing ability in the presence of immune serum.

Hyperimmune Serum. Guinea pigs were inoculated intraperitoneally with a 10% suspension of active virus derived from mouse brain. A total of 8 injections was given at intervals of 3 or 4 days over a period of 3 weeks and consisted of 3 inoculations of 0.05 cc, 4 of 0.1 cc, and 1 of 0.2 cc. Four days after the last injection they were bled by cardiac puncture and the sera pooled and frozen in small tubes at −70°C.

Complement Fixation. Serum of guinea pigs served as complement. It was titrated in the presence of the amount of antigen used in the test. The unit of complement was selected as the highest dilution which in the presence of antigen caused complete lysis of the hemolytic system. One and one-half such units of complement were chosen for most tests.

The sera from immune guinea pigs were inactivated at 56°C for 30 minutes. Two-fold dilutions were employed in 0.2 cc amounts. No great difference in titer obtained whether single or multiple pipettes were used in preparing the dilutions of serum. 0.2 cc of complement was then added followed by 0.2 cc of the clarified 10% suspension of virus. The tubes were incubated in the water bath at 37°C for one hour. 0.3 cc of a suspension of sensitized sheep cells representing a concentration of 2% by volume of packed cells and 2 units of anti-sheep cell hemolysin was then added and the tests read after exactly 15 minutes' incubation at 37°C.

By this method it was found that specific complement fixation uncomplicated by cross reactions could be obtained with antigens prepared from the viruses of Eastern and Western equine encephalomyelitis and homologous immune sera. Antigens prepared in the same manner from normal chick embryos failed to react with either serum.

In Table I are presented the results of a typical test. Complete fixation was usually obtained with dilutions of homologous serum as high as 1:64.

Effect of Inactivation of Virus upon the Antigenic Activity. The highly infectious antigens of virus titering 10^{-8} for the Eastern type and 10^{-6} for the Western type were exposed to ultraviolet irradiation at a distance of 4 cm from an 8-watt General Electric

[1] Howitt, B. F., *J. Immunol.*, 1937, **33**, 235.

[2] Howitt, B. F., abstracted in *J. Bact.*, 1938, **36**, 52.

[3] Casals, J., and Palacios, R., *Science*, 1941, **94**, 330.

[4] Casals, J., and Palacios, R., *J. Exp. Med.*, 1941, **74**, 409.

[5] Casals, J., *Am. J. Pub. Health*, 1941, **31**, 1281.

[6] Howitt, B. F., *J. Immunol.*, 1943, **47**, 293.

[7] Havens, W. P., Watson, D. W., Green, R. H., Lavin, G. I., and Smadel, J. E., *J. Exp. Med.*, 1943, **77**, 139.

[8] Mohler, W. M., *J. Am. Vet. Med. Assn.*, 1939, **94**, 39.

TABLE I.
Complement Fixation Tests with Eastern and Western Equine Encephalomyelitis Viruses and Guinea Pig Hyperimmune Serum

Serum	Antigen	Original dilution of serum 1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	Controls: Antigen Anti-comp.	Antigen Hemol.	Serum Anti-comp.	Serum Hemol.
WEE	WEE	4*	4	4	4	4	4	2	0	0	4	0	4
	EEE	0	0	0	0	0	0	0	0	0	4	0	4
	Normal	0	0	0	0	0	0	0	0	0	4	0	4
EEE	WEE	0	0	0	0	0	0	0	0	0	4	0	4
	EEE	4	4	4	4	4	4	1	0	0	4	0	4
	Normal	0	0	0	0	0	0	0	0	0	4	0	4

* 4 = no hemolysis. 0 = complete hemolysis.

germicidal lamp.† Irradiation for more than 45 minutes destroyed completely the infectivity of the Eastern type of virus but the complement-fixing ability was unchanged even after 120 minutes' exposure. Furthermore, no obvious decrease in antigenic titer occurred after 10 weeks' storage at 4°C.

The infectivity of the Western antigen was destroyed after 20 minutes' irradiation while complement fixation was obtained with antigen irradiated as long as 30 minutes. Loss of potency occurred with prolonged irradiation as well as irregularity in the anti-complementary power of the antigen.

Improved methods of preparation, the effect of other inactivating procedures and the efficacy of these antigens with human sera are being studied.

† Further details will be described in a later communication.

14606 P

Slide Agglutination Test for Rapid Diagnosis of Pre-Eruptive Typhus Fever.

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A specific antigen occurs in the serum of patients during the first few febrile days of typhus fever.[1] Approximately 60% of 107 typhus patients studied during the pre-eruptive stage had sufficient amounts of this specific immune substance in their bloods to be detected by the complement fixation technic; furthermore, the antigen was absent from sera of 57 patients with typhoid, dysentery, and pneumonia. The circulating antigen was no longer demonstrable after specific antibodies made their appearance, *i.e.*, 6th to 9th day.[1] Although satisfactory results were obtained with the complement fixation technic, it seemed desirable to employ a simpler and more rapid method for demonstrating the antigen in the blood of patients with early typhus.

Various attempts were made to develop a slide agglutination test sufficiently sensitive for the detection of the comparatively small amounts of antigen present in blood of typhus patients. For this purpose a number of particulate substances were added to typhus antigens, usually Cox type vaccine, which were

[1] Drobyshevskaya, A. I., and Smorodintzeff, A. A., *J. Epid. and Microbiol.* (Moscow), 1942, No. 1.

employed in preliminary studies. The addition of neither animal charcoal, erythrocytes nor *B. prodigiosum* had the desired effect. On the other hand, suspensions of carmine, of indigo, and of other water-insoluble aniline dyes when mixed with rickettsial material gave results which suggested their usefulness in testing for typhus antigen in patients' sera. Carmine has been used most extensively in our work.

Method. The technic of the slide agglutination test in current use is as follows: Chemically pure carmine is ground to a fine powder in a mortar and a 2% suspension is prepared by the gradual addition with constant trituration of freshly distilled water. The suspension is shaken for 10 minutes in a bottle with glass beads. Large particles are removed by short centrifugation at slow speed. The suspension is allowed to stand 24 hours and then centrifuged in order to sediment most of the carmine particles: the supernatant fluid is decanted and discarded. The sediment is resuspended to the original volume in a 0.25% solution of NaCl. The dye is now ready for use.

Serum to be tested for the presence of typhus antigen is diluted 1 to 5 with distilled water and ¼ volume of carmine suspension is added. The mixture is shaken for 5-10 minutes then lightly centrifuged (500 r.p.m. for 5 minutes) after which most of the supernatant fluid is decanted and the sediment resuspended in the residual fluid. While such antigen-laden particles of carmine are specifically agglutinated by the human serum rich in antibody, preliminary experiments have indicated that the intensity of reaction is increased several-fold if the antibody is used not as such but also adsorbed on particles of carmine. In the actual test, two drops of the suspension of carmine, laden with serum collected during the acute phase of the disease (antigen), are placed separately on a glass slide and to one of them is added a drop of a suspension of carmine particles laden with antibody-containing serum, and to the other, as control, is added a suspension of particles to which negative serum was adsorbed. Additional control tests of the obvious type are carried out. Agglutination becomes visible macroscopically within a few minutes.

It is important in the test to employ solutions with relatively low concentrations of electrolytes in order to avoid spontaneous agglutination of carmine particles; even so, certain lots of carmine are unusable because of nonspecific aggregation. Tests with acute phase sera stored for longer than 5 days are generally unsatisfactory.

Results. Over 100 sera from early cases of typhus fever and other infectious diseases were tested for typhus antigen by the complement fixation technic[1] and the carmine particle agglutination method. The immunological specificity of the results obtained by the two methods were comparable.

Preliminary studies suggest that instead of serum, whole blood obtained by finger prick can be used in the test for antigen. The blood is lysed by dilution with distilled water and the test carried out as above.

The presence of typhus antibodies in sera of convalescent patients and hyperimmune guinea pigs and rabbits is readily demonstrated by this same slide agglutination method. Antigen for use in this procedure is prepared by adding an equal volume of 2% carmine suspension to appropriately diluted (usually 1/20) typhus vaccine of the Cox type. Serum to be tested for antibody is prepared as described above.

Summary. A specific antigen found in the serum of typhus fever patients during the pre-eruptive stage of typhus fever can be demonstrated by a slide agglutination test which employs sensitized dye particles.

14607

Determination of Biotin with *Lactobacillus arabinosus*.

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Snell and Wright reported that biotin is an essential growth factor for *Lactobacillus arabinosus*.[1] It was stated that the microbiological determination of biotin may be accomplished through the use of the same basal medium employed in the microbiological determination of nicotinic acid, provided that biotin is excluded and nicotinic acid is added. Since then several procedures have appeared for the microbiological determination of biotin with *Lactobacillus casei*.[2,3,4]

The growth factor requirements of *L. arabinosus* are less complex than those of *L. casei*, since luxuriant and rapid growth of *L. arabinosus* may be obtained in a medium composed only of certain purified amino acids, glucose, purines and pyrimidines, inorganic salts, and several synthetic members of the vitamin B complex. Growth stimulation by an eluate factor from tomato juice of unknown composition[5] occurs only under certain conditions where the basal medium and the medium for inoculation are prepared from very nighly purified constituents.[6] On the other hand, *L. casei* is stimulated by a variety of unknown factors and, in addition, requires the presence of "folic acid."[7] Desthiobiotin has an antibiotin activity for *L. casei* but it does not affect the growth of *L. arabinosus*.[8,9] Difficulty has been reported in the preparation of an adequate basal medium for the determination of biotin by one of the methods using *L. casei* but certain improvements in procedure have been described.[4]

Saccharomyces cerevisiæ has been used widely as a test organism in the determination of biotin.[10] It has been shown, however, that yeast responds to peroxide-treated biotin,[11] as well as to certain forms of biotin which do not combine with avidin (vitamers[12]) and while such yeast methods may be useful in studies of biotin metabolism they may be criticised because of lack of specificity.

Clostridium butylicum[13] and *Staphylococcus aureus*[14] have been recommended as test organisms for the determination of biotin. These bacteria have not, however, been widely used.

Since *L. arabinosus* has been used successfully for the determination of biotin in these laboratories for some time, the details of the procedure are presented here.

Procedures for the use of *L. arabinosus* in the microbiological determination of pantothenic acid, *p*-aminobenzoic acid, nicotinic

1 Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, 1941, **139**, 675.

2 Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, 1942, **27**, 1086.

3 Shull, G. M., Hutchings, B. L., and Peterson, W. H., *J. Biol. Chem.*, 1942, **142**, 913.

4 Shull, G. M., and Peterson, W. H., *J. Biol. Chem.*, 1943, **151**, 201.

5 Kuiken, K. A., Norman, W. H., Lyman, C. M., and Hale, F., *Science*, 1943, **98**, 266.

6 McMahan, J. R., and Snell, E. E., *J. Biol. Chem.*, 1944, **152**, 83.

7 Mitchell, H. K., Snell, E. E., and Williams, R. J., *J. Am. Chem. Soc.*, 1941, **63**, 2284.

8 Dittmer, K., Melville, D. B., and du Vigneaud, V., *Science*, 1944, **99**, 203.

9 Lilly, V. G., and Leonian, L. H., *Science*, 1944, **99**, 205.

10 Snell, E. E., Eakin, R. E., and Williams, R. J., *J. Am. Chem. Soc.*, 1940, **62**, 175.

11 Nielsen, E., Shull, G. M., and Peterson, W. H., *J. Nutrition*, 1942, **24**, 523.

12 Burk, D., and Winzler, R. J., *Science*, 1943, **97**, 57.

13 Lampen, J. O., Kline, A. A., and Peterson, W. H., Proc. Am. Soc. Biol. Chem., *J. Biol. Chem.*, 1941, **140**, lxxiv.

14 Porter, J. R., and Pelczar, M. J., *Science*, 1940, **91**, 576.

acid, and a variety of amino acids have appeared.

Procedure. The details of the procedure resemble those described for other microbiological methods involving lactic acid bacteria.

L. arabinosus 17-5* is carried in 1% glucose, 1% yeast extract, 1.5% agar stabs. Transfers from previous stock cultures are made monthly. The fresh transfers are incubated at 30°C for 3 days and then are held in the refrigerator.

One day prior to the procedures leading to the determination of biotin an inoculum is prepared by transfer of the organism from stock culture to a medium of 1% glucose and 1% yeast extract. The latter is incubated at 30°C for 18-36 hours before use. After growth the cells are centrifuged and washed once with sterile saline and a faintly visible saline suspension prepared for seeding the unknown and the standard tubes.

The constituents of the basal medium are shown in Table I. It is convenient to prepare, in one solution of double strength, all the ingredients of the medium with the exception of glucose and the synthetic vitamins. This solution may be preserved under benzene without previous heat sterilization. A stock solution is prepared containing in each 100 ml: thiamin chloride, riboflavin, pantothenic acid, nicotinic acid, 4 mg of each; pyridoxine hydrochloride, 8 mg; and para-aminobenzoic acid, 0.4 mg.

TABLE I.
Basal Medium.

Hydrochloric acid-hydrolyzed, norite-treated, vitamin-free casein (16)	0.5	%
Tryptophane	0.01	"
Cystine*	0.01	"
Glucose	2.0	"
Sodium acetate (anhydrous)	0.6	"
K_2HPO_4	0.05	"
KH_2PO_4	0.05	"
NaCl	0.001	"
$MgSO_4 \cdot 7H_2O$	0.02	"
$FeSO_4 \cdot 7H_2O$	0.001	"
$MnSO_4 \cdot 4H_2O$	0.001	"
Adenine sulfate†	5	ppm
Guanine hydrochloride†	5	"
Xanthine†	5	"
Uracil†	5	"
Thiamine	1.0	"
Calcium pantothenate	1.0	"
Pyridoxin hydrochloride	2.0	"
Riboflavin	1.0	"
Nicotinic acid	1.0	"
Para aminobenzoic acid	0.1	"

* Dissolved separately with the aid of a small amount of concentrated hydrochloric acid.

† Dissolved separately by heating with hydrochloric acid.

* Cultures of this organism may be secured from the American Type Culture Collection, Georgetown University Medical School, Washington, D.C., where it is listed as No. 8014.

Scrupulously clean pyrex bacteriological test tubes are used and are supported in a wire rack. Appropriate amounts of a biotin solution are pipetted into a series of tubes to establish a standard curve representing the response of *L. arabinosus* to varying amounts of biotin. Various amounts of the materials to be assayed are pipetted into additional tubes. All tubes are then diluted to 5 ml with distilled water. An amount of medium (double strength) sufficient to supply 5 ml for each assay tube is supplemented with 4 g of glucose and 5 ml of vitamin solution per 100 ml. Five ml of the complete medium is then added to each assay tube. The tubes are plugged with cotton and the test autoclaved at 15 lb pressure for 15 minutes. After cooling to room temperature each tube is seeded aseptically with one drop of the bacterial suspension for inoculation. The test is incubated at 30-33°C for approximately 72 hours. Titration of the lactic acid produced, using bromthymol blue as the indicator, is used as a measure of the response of the organism to biotin. From the response of *L. arabinosus* to pure biotin (Fig. 1), which must be determined with every test, the biotin content of the unknown samples is calculated. We have found it convenient to assay unknown materials at 4 different levels. In a successful assay there should be good agreement between the four levels of unknown material assayed.

Results. Comparable results obtained when a variety of natural materials were microbiologically assayed for biotin by the proposed procedure employing *Lactobacillus arabinosus*, and by the biotin procedure of Landy and Dicken[2] with *Lactobacillus casei* are given in Table II. Both procedures gave similar results when applied to materials autoclaved with acid and to some untreated materials. With certain other water-soluble materials, particu-

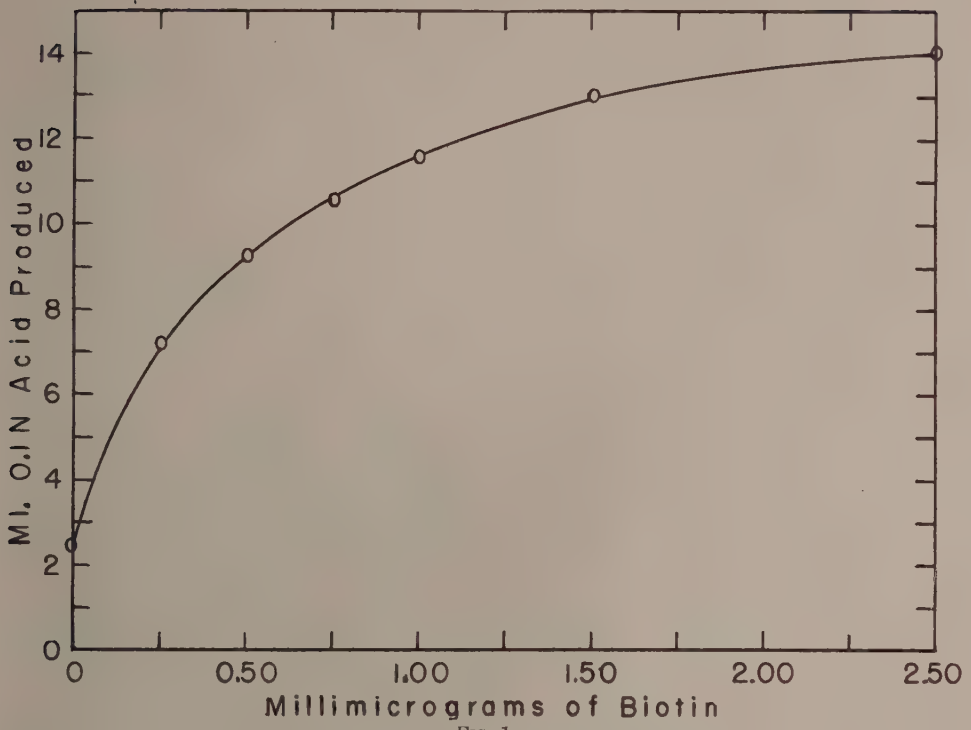

FIG. 1.
The response of *Lactobacillus arabinosus* to added biotin.

TABLE II.
Biotin Content of Various Materials as Determined by *L. arabinosus* and by *L. casei*.

Material	Biotin content	
	L. arabinosus μg/g or ml	*L. casei* μg/g or ml
Curbay powder, untreated	3.7	3.9
Valentine's meat extract, untreated	.038	.038
Bacto peptone, untreated	.15	.16
" " acid autoclaved	.17	.17
Proteose peptone, untreated	.11	.44
" " acid autoclaved	.37	.40
Urine, untreated	.032	.032
Purina chow, acid autoclaved	.19	.24
Yeast extract, untreated	.090	1.6
" " acid autoclaved	1.5	1.8
Malt extract, untreated	.045	.065
Trypsinized vitamin-free casein	.00081	.010
Grass juice powder, untreated	.34	.53
Tryptone, untreated	.16	.22
Klim, untreated	.16	.20
Liver powder 1:20, untreated	.22	.27
" " 1:20, acid autoclaved	.29	.21

larly autolyzed or enzyme-treated substances, higher values were obtained with *L. casei*. Acid hydrolysis (6 N H_2SO_4 at 15 lb pressure for 1 hour) did not affect the amount of biotin found with *L. casei* but increased the observed biotin content when determined with

L. arabinosus to a value equal to that found with *L. casei.* It would appear that there exists in certain enzymatically prepared materials a form of biotin which may be utilized for growth and acid production by *L. casei* but not by *L. arabinosus.* Using yeast extract as an example, it was possible to show that this form of biotin, available to *L. casei* but not to *L. arabinosus,* was avidin-combinable. Acid autoclaving converts this form of biotin to one used equally well by the two organisms. This need not seriously interfere with the use of *L. arabinosus* as an assay organism for biotin since it is well known that there exist in many natural materials combined forms of biotin which are not available to bacteria. Preliminary acid hydrolysis has become an accepted procedure before biotin determination by any method.

The recoveries obtained when biotin was added to several naturally occurring materials are given in Table III. Quantitative recovery of biotin was readily obtained. It is believed that better agreement at varying assay levels for the biotin content of various materials is obtained with the *L. arabinosus* method than is possible with the *L. casei* method of Landy and Dicken.[2]

Since pimelic acid is capable of being utilized in lieu of biotin by the diphtheria bacillus,[15] the ability of *L. arabinosus* to utilize similar dicarboxylic acids was studied. Adipic, pimelic, suberic, azelaic, and sebacic acids were found to possess no biotin-like activity for *L. arabinosus.*

TABLE III.
Recovery of Biotin* Added to Various Materials.

Sample	Biotin content μg/g or ml	Recovery %
Proteose peptone†	0.11	100
Bacto peptone†	0.15	100
Malt extract†	0.050	100
Rat liver‡	0.48	101

* Biotin added to the final solution for assay in an amount which approximately doubled that originally present.

† A solution of these materials assayed without previous treatment.

‡ Autoclaved with 6 N H_2SO_4 at 120°C for 1 hour before determination.

Summary. The microbiological determination of biotin, using *Lactobacillus arabinosus* as the test organism, was studied. The existence of a water-soluble, avidin-combinable form of biotin in certain enzymatically prepared materials which is available to *L. casei,* but not to *L. arabinosus,* was demonstrated. When natural materials are autoclaved with 6 N H_2SO_4, prior to determination of their biotin content, comparable results are obtained with the *L. arabinosus* method and with a method using *L. casei.*

[15] du Vigneaud, V., Dittmer, K., Hague, E., and Long, B., *Science,* 1942, **96**, 186.

[16] *The University of Texas Publication,* 1941, **4137**, 82.

14608

Methionine Deficiency in Yeast Protein.

A. A. KLOSE AND H. L. FEVOLD.

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The present shortage of proteins has stimulated interest in the production of yeast for use primarily as a source of food (or feed) protein. Work now in progress in this laboratory[1,2] involves the development of methods to produce torula yeasts grown on molasses and fruit wastes, and the evaluation of these yeasts as a source of feed protein.

* One of four regional research laboratories operated by the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

[1] Stubbs, J. J., Noble, W. M., and Lewis, J. C., in press.

[2] Lewis, J. C., Stubbs, J. J., and Noble, W. M., in press.

TABLE I.
Composition of Diets in %.

Diet	Basal mixture	Starch-sucrose 50-50	Yeast extr.	Casein	Brewers' Yeast	Torula Yeast	% crude protein
I	65	19	≅5	16	—	—	13.4
II	65	5	—	—	30	—	13.2
III	65	10	—	—	—	25	13.3

Considerable work, much of it quite recent, has been carried out on the feeding value and amino acid composition of yeast protein. Hock[3] demonstrated the inability of yeast to replace completely a fish meal-casein mixture in diets of 9.3% crude protein when fed to growing rats. Csonka[4] has reported values for tryptophane, histidine, lysine, and arginine in yeast which would constitute adequate amounts of these essential amino acids for the rat, based on the requirements tentatively set by Rose[5] and the presence of at least 13.5% of crude yeast protein in the diet.

Since the work reported here, dealing with the evaluation of yeast protein, was completed, another set of amino acid values has come to our attention. Melnick[6] has cited data obtained from a book in press by Block and Bolling[7] as the source of analyses of yeast powder for all of the 10 essential amino acids. At a 13.5% crude protein level, these values represent adequate amounts for the rat, except in the case of methionine, which would be present to the extent of only 0.27% compared with a requirement of 0.6%. This information substantiates the results of our experiments. To our knowledge no feeding test demonstrating the lack or unavailability of sufficient methionine in yeast for the growing rat has been reported.

Two samples of dried yeast—II, a commercial brewers' yeast, and III, a pilot-plant product of *Torulopsis utilis* grown on molasses—have been fed to 38-day-old rats as the sole source of dietary protein and at a crude protein (6.25 x % nitrogen) level equivalent to that of a 16% commercial casein diet. No corrections have been made for the amount of non-protein nitrogen (purines and pyrimidines) which may represent as much as 15% of the total nitrogen of yeast. However, the conclusions drawn from the experimental data are not changed essentially by the presence of such amounts of non-protein nitrogen.

The basal mixture for the diets described in Table I contained, as percent of the complete diet: sucrose 25, corn starch 25, Wesson (cottonseed) oil 10, McCollum's salt mixture (No. 185) 4, and U.S.P. cod liver oil 1. Vitamin contents, as mg per 100 g of complete diet, were: choline chloride 50, thiamin chloride 0.2, riboflavin 0.5, pyridoxin 0.2, calcium pantothenate 2.5, and nicotinic acid 1.0.

The yeast extract was prepared by extract-

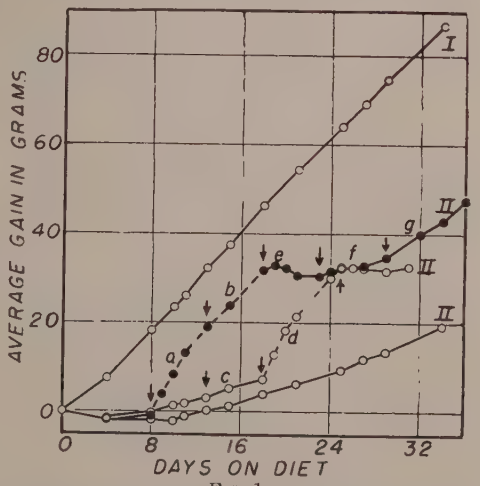

FIG. 1.

The effect of methionine and other amino acid supplements on the growth rate of rats fed brewers' yeast. A change in diet is indicated by a vertical arrow. Amino acid supplements to the brewers' yeast diet II were: (a) methionine, threonine, valine, leucine, and isoleucine; (b) methionine, threonine, and valine; (c) leucine and isoleucine; (d) methionine; (e) threonine and valine; (f) threonine, valine, leucine, and isoleucine; and (g) cystine. I is a reference diet (16% casein).

3 Hock, A., *Biochem. Z.*, 1942, **311**, 385.

4 Csonka, F. A., *J. Biol. Chem.*, 1935, **109**, 703.

5 Rose, W. C., *Science*, 1937, **86**, 298.

6 Melnick, D., *Wallerstein Laboratories Communications*, 1943, **6**, 167.

7 Block, R. J., and Bolling, D., in press.

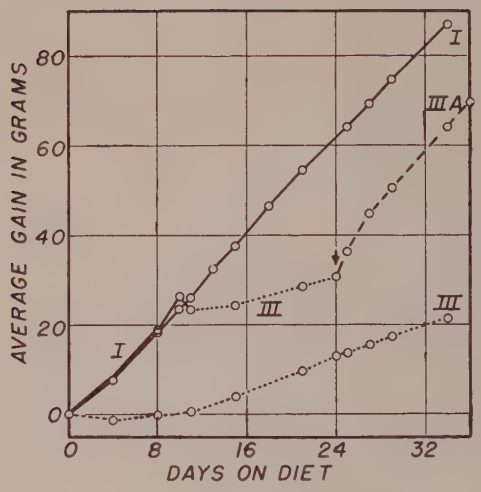

FIG. 2.

The effect of a methionine supplement on the growth rate of rats fed torula yeast. I—16% casein diet; III—25% torula yeast; IIIA—25% torula yeast + 0.5% methionine. A change in diet is indicated by a vertical arrow.

ing 2400 g of brewers' yeast II with 3 successive 6-liter volumes of 50% aqueous methyl alcohol. The extract was concentrated *in vacuo* to a thick syrup and fed in diet I at a level equivalent to 5% whole yeast, in order to make the 3 diets more comparable with respect to all factors except protein.

Diets II and III were supplemented with commercial grades of amino acids as indicated in Fig. 1 and 2. Each of the amino acids was fed at a level of 0.5%, mixed into the whole diet, with the exception of cystine, which was fed at a 1% level.

Six groups of rats were made up of equal numbers of males and females selected from the same litters of approximately equal age, with an average initial weight ranging from 72 to 75 g. A group of 32 rats was placed on diet 1 as a positive control (growth curve in both Fig. 1 and 2); the 5 other experimental groups each contained 16 rats. As indicated in Fig. 1, 3 groups were fed diet II, and two of these were given successively different combinations of amino acids. The broken lines in Fig. 1 indicate where the diet contains a supplement of 0.5% methionine. Fig. 2 shows growth curves for groups on diets I and III, and for one group started on diet I, then changed to diet III, and finally supplemented with 0.5% methionine (heavy broken line).

The rats had been fed a stock diet of Purina Dog Chow supplemented with lettuce and cod liver oil prior to the feeding of the experimental diets. The experimental diets were fed *ad libitum* and feed consumption in the 3 groups, which ranged from 8 to 11 g per rat per day, was correlated roughly with the gains in weight. No deaths occurred during the 5-weeks experimental period.

Both samples of yeast failed to support even moderate growth when fed as the sole source of protein. The addition of 0.5% *dl*-methionine alone to these yeast diets resulted in a growth rate equal to that obtained with a diet containing 16% casein. It appears from these results that, compared with casein and at the levels fed, yeast is seriously deficient for the growing rat in only one amino acid, methionine. (See Fig. 1 and 2.)

Womack and Rose[8] have shown that in diets containing 0.4% cystine, only one-sixth of the requirement of 0.6% methionine for optimum growth can be satisfied by the cystine. The yeast may be estimated to have contributed 0.1[4] to 0.3%[9] cystine to our diets, an amount which theoretically should preclude any significant increase in growth rate due to added cystine.

The methionine deficiency in yeast might conceivably involve the question of the availability of the methionine present, as has been found true for soybean protein.[10,11] However, the digestibility of yeast both *in vivo*[6] and *in vitro*[12] has been measured and found to be quite high. Further work is in progress, both with the rat and with the chick, and will be reported at a later date.

Summary. Brewers' yeast and torula yeast grown on molasses, when either was fed as the sole source of protein to growing rats, were

8 Womack, M., and Rose, W. C., *J. Biol. Chem.*, 1941, **141**, 375.

9 Prunty, F. T. G., *Biochem. J.*, 1933, **27**, 387.

10 Hayward, J. W., and Hafner, F. H., *Poultry Sci.*, 1941, **20**, 139.

11 Almquist, H. J., Mecchi, E., Kratzer, F. H., and Grau, C. R., *J. Nutrition*, 1942, **24**, 385.

12 von Soden, O., and Dirr, K., *Biochem. Z.*, 1942, **312**, 252.

found to be lacking in sufficient available methionine to produce growth equal to that elicited by an equivalent amount of casein. It appears that this fact is one limiting factor which must be considered in evaluating yeast protein as a replacement for animal proteins.

14609

Penicillin Sensitivity of Strains of Non-hemolytic Streptococci Isolated from Cases of Sub-acute Bacterial Endocarditis.

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It is generally recognized that different organisms vary greatly in their sensitivity to penicillin and that even different strains of the same organism exhibit wide variations in susceptibility. In the treatment of human infections the importance of determining the sensitivity of the infecting strain is therefore obvious. In view of the apparent success which has recently been achieved in the treatment of cases of sub-acute bacterial endocarditis with penicillin,[1,2] information on the penicillin sensitivity of strains isolated from this disease is particularly desirable.

Fifty strains of non-hemolytic streptococci obtained from cases of sub-acute bacterial endocarditis were studied.

Gross Description. Of 50 strains examined:

19 were described as "viridans"
12 " " " "indifferent"
3 " " " "resembling enterococci"
2 exhibited 2 types of colonies
1 was described as "weakly hemolytic"
13 were not described, other than as "non-hemolytic"

Serological Grouping. Thirty-nine strains were examined serologically according to Lancefield's procedure. Of these, 10 fell into definite groups as follows:

Group B	1 strain
" C	2 strains
" D	1 strain
" F	2 strains
" H	4 "

In addition, 6 strains showed an antigenic relationship to one or other of the groups but could not be definitely classified. Twenty-three strains showed no relationship to any of the groups for which they were tested. Eleven strains were not tested serologically.

Penicillin Sensitivity. The sensitivity of non-hemolytic streptococci was determined by the following procedure:

The sensitivity of each strain was compared with that of a standard strain of hemolytic streptococcus (C203MV). It had been shown previously that from 0.01 to 0.03 Oxford units are required to inhibit the growth of a 10^{-2} dilution of a 15-hour rabbit's blood broth culture of this strain. Such a dilution contains from 2,500,000 to 3,000,000 organisms. In other experiments it had been shown that the *in vitro* activity of penicillin is influenced by the number of organisms present and by the rate of growth of the organisms. The unknown strains were therefore tested under growth conditions comparable with those of the standard strain. The cultures to be examined were grown for 15 hours in rabbit's blood broth, the original seeding being such as to produce growth of approximately the same density as that of a 15-hour culture of strain C203MV. If the unknown strain grew too heavily the 15-hour culture was diluted with broth to a turbidity equivalent to that of the standard. If the unknown strain grew too lightly, a 10^{-1} dilution was used for the test instead of a 10^{-2} dilution. If extremely heavy or very sparse growth occurred, a more suit-

1 Loewe, L., Rosenblatt, P., Greene, H. J., and Russell, M., *J. A. M. A.*, 1944, **124**, 144.

2 Dawson, M. H., and Hobby, G. L., *J. A. M. A.*, 1944, **124**, 611.

able 15-hour culture was obtained by using a more appropriate seeding.

Two sets of serial dilutions of penicillin in plain broth containing 8, 4, 2, 1, 0.5, 0.25, 0.12, 0.06, 0.03, 0.015 units per cc respectively were then prepared. To the first set was added a sufficient amount of a 15-hour rabbit's blood broth culture of the standard strain to give a final dilution of 10^{-2}. To the second set was added a similar amount of the unknown strain. Both sets were then incubated at 37°C overnight and the titers read the following day.

The least amount of penicillin (units per cc) which inhibited growth as evidenced by absence of turbidity, was accepted as the "titer." The "titer" of the unknown strain was compared with that of the standard strain and the relative sensitivity of the 2 strains was recorded. For example, if 0.03 units were required to inhibit growth of the standard strain and 0.06 units the unknown strain, the unknown strain was considered to be one-half as sensitive.

The sensitivity of 41 strains compared with that of the standard strain of hemolytic streptococcus was found to be as follows:

1 strain was twice as sensitive
8 strains were equally as sensitive
23 strains were ¼ to ½ as sensitive
4 strains were ⅛ as sensitive
2 strains were $\frac{1}{16}$ as sensitive
1 strain was $\frac{1}{64}$ as sensitive
2 strains were highly resistant

Analysis of the sensitivity of the strains according to the gross description revealed the following:

(a) Of 19 strains described as "viridans"
1 was twice as sensitive
2 were equally as sensitive
10 were ¼ to ½ as sensitive
2 were $\frac{1}{16}$ as sensitive
1 was $\frac{1}{64}$ as sensitive
1 was resistant
2 were not tested for sensitivity

(b) Of 12 strains described as "indifferent"
3 were equally as sensitive
6 were ¼ to ½ as sensitive
3 were not tested

(c) Of 3 strains described as "resembling enterococci"
1 was equally as sensitive
1 was ½ as sensitive
1 was resistant

Analysis of the sensitivity of strains according to serological grouping showed that of the 10 strains which fell into definite serological groups, all except one were either equally as sensitive or ¼ to ½ as sensitive as the standard strain. One strain belonging to Group H was ⅛ as sensitive.

Previous work in this laboratory has shown that the majority of strains of staphylococci isolated from human infections are from 2 to 8 times more resistant to penicillin than the standard strain of hemolytic streptococcus employed in the present investigation.

Conclusions. With occasional exceptions strains of non-hemolytic streptococci isolated from cases of sub-acute bacterial endocarditis are sensitive to penicillin *in vitro*. The degree of sensitivity is approximately the same as that shown by strains of staphylococci isolated from human infections but is not as great as that exhibited by hemolytic streptococci. The degree of sensitivity of any particular strain does not correspond with the cultural or serological properties of the strain. In the treatment of each individual case, the sensitivity of the infecting strain should be determined as a guide to appropriate therapy.

14610

An Optical Recording Rotameter for Measuring Blood Flow.*

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The rotameter, described in a previous communication,[1] is a simple and reliable instrument with which measurements of average rate of blood flow may be made in acute experiments. The instrument is a variable area type meter and consists of a vertical tube having a tapering bore in which a freely movable metal "float" varies in its vertical position with the rate of blood flow through the tube.

Depending upon the flow range to be covered, the capacity and sensitivity of the same rotameter may be modified by using "floats" of different shapes, diameters, specific gravities or weights. For more extreme blood flow ranges, tubes with bores of different diameters or degrees of taper may be used.

While the commercially available rotameters have been found satisfactory for measuring blood flow from as low as 0.5 cc/min. to at least 1200 cc/min., they were designed primarily for industrial use. Consequently certain features in design were modified in preliminary models to permit more advantageous use of the rotameter in physiological work. The regular precision bore Pyrex glass rotameters are considerably longer than necessary and, although quite rugged, are still subject to accidental breakage. These disadvantages were overcome by making the rotameter tubes of ½-inch to ⅝-inch Lucite rod with an over-all length of 6-10 cm. The central hole was reamed with a standard taper-pin reamer, the size of which was chosen to give the instrument the desired sensitivity and range. The surface was finished by polishing with magnesia. Metal "floats" of appropriate size and shape were turned on a lathe. The threaded or tapered fittings at the ends of the tube were constructed to accommodate cannulæ attachments. An arbitrary scale of millimeter graduations was cut into the Lucite for notation and calibration of the height of the "float."

Although these earlier instruments were quite satisfactory from the standpoint of being accurate, convenient, and relatively unbreakable, they still required visual notation of the height of the float and periodic calibration of several points during prolonged experiments to correct for any major change in blood viscosity. These objections were circumvented by developing further changes and additions. In the most recent design, the tapered portion of the tube was shortened to 11-12 mm. The shape of the "float" was modified so as to eliminate almost entirely shifts in calibration due to changes in blood viscosity. An electrical unit was developed with which the height of the "float" could be determined and blood flow automatically and optically recorded on sensitized paper. With the use of this unit, a continuous recording of blood flow could be made simultaneously with blood pressure, ECG, or other tracings. The electrical unit is small, can be made from standard radio parts, and is described in a companion paper.[2] The instrument has been used thus far to record coronary and peripheral arterial blood flow as well as venous flow.[3] In Fig. 1 is a photograph of an original record of coronary flow obtained with the recording rotameter.

A sectional view of the rotameter proper is shown in Fig. 2. The body of the instrument is made from ½-inch Lucite and is constructed in 3 pieces which are screwed together. The lumen of the lower piece is tapered as shown. The lumen of the central (cross hatched)

* The expenses for this investigation were defrayed by a grant from the Commonwealth Fund.

† Department of Physics, Case School of Applied Science, Cleveland, Ohio.

1 Gregg, Donald E., Shipley, R. E., Eckstein, R. W., Rotta, A., and Wearn, J. T., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 267.

2 Crittenden, E. C., Jr., and Shipley, R. E., *Rev. Scient. Inst.*, in press.

3 Gregg, Donald E., and Shipley, R. E., *Amer. Jour. Physiol.*, in press.

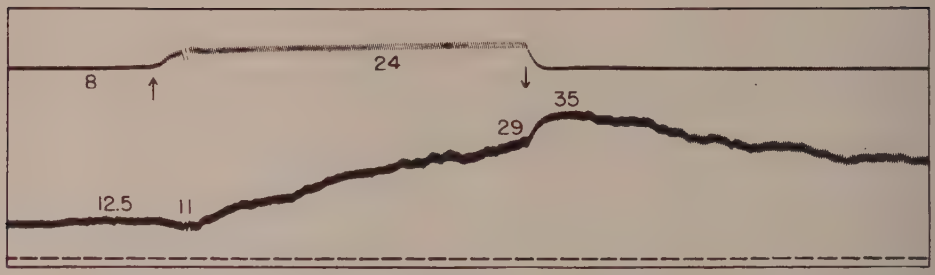

FIG. 1.

Photograph of original record showing the effect of mean right ventricular pressure elevation on right coronary inflow (perfusion pressure kept constant). Upper curve, mean right ventricular pressure with values in mm Hg. indicated by numbers adjacent to curve. Lower curve, mean right coronary inflow as measured by the recording rotameter unit with values in cc/min. indicated by numbers adjacent to curve. (↑) Pulmonary artery constricted, (↓) pulmonary artery released. Time, 5 seconds. (For interpretation of experiment, see reference.[3])

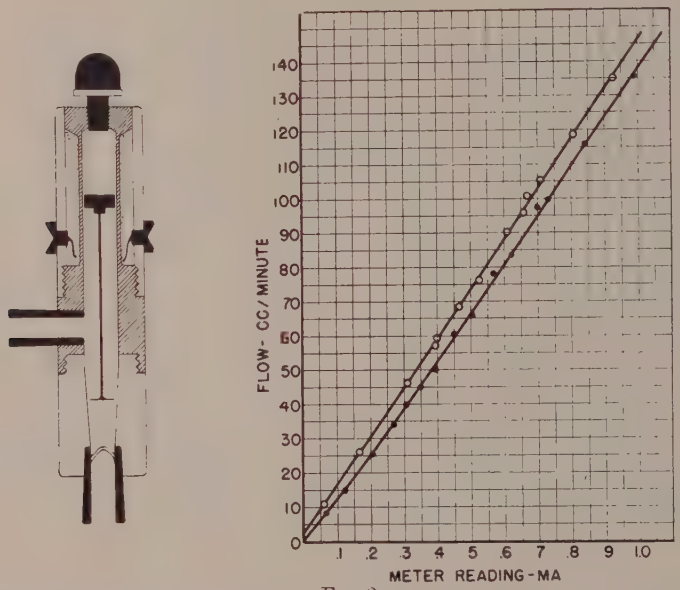

FIG. 2.

Sectional view (actual size) of rotameter unit. Description in text. Graph showing relative linearity of calibrations and comparatively small shift in calibration due to large change in fluid viscosity. Open circles, calibration obtained with water (spec. visc. = 1). Closed circles, calibration obtained with acacia solution having a viscosity approximating that of blood (spec. visc. = 4.5).

piece is drilled straight and polished. Around its upper part, and separated from the lumen by a thin shell of Lucite .020 inches thick, is wound .0025 inches enameled copper wire with decreasing number of turns from top to bottom. Over the upper part of the rotameter is fastened a Lucite sleeve which adds rigidity and protects the coil. Melted paraffin may be forced under the sleeve to give further mechanical and electrical protection to the wire. Slightly hollowed contacts fixed to the outer sleeve serve as the external terminals of the coil as well as the supporting points for the unit when in use (*cf.* Fig. 3).

The "float" is a soft iron cylinder connected to a phosphor-bronze wire, at the bottom of which is soldered a thin (.007-inch) brass disc, all of which are chrome plated.

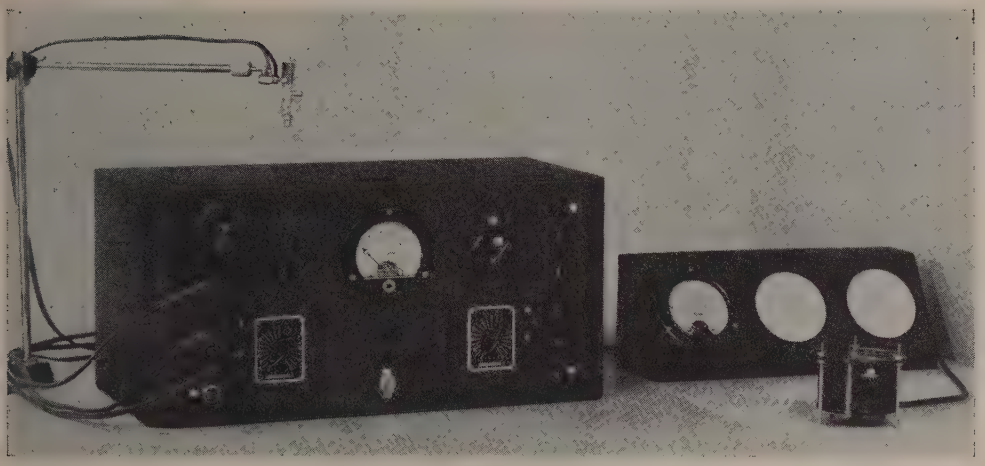

Fig. 3.

Photograph of apparatus. A, rotameter unit held in support as used. B, cabinet containing electrical unit with which balancing, sensitivity, and recording adjustments are made. Monitor meter mounted in cabinet face. C, portable monitor meter with long lead connection. D, recording meter (0-7.5 MA) mounted on 3 adjustable legs. Hole cut from side of meter to permit reflection of light beam from 3 mm x 3 mm mirror mounted to counterbalance end of meter hand.

A "stop," soldered to the tapered cannula fitting at the bottom, establishes the zero position of the "float." A tapered plug at the top of the rotameter can be removed to allow the escape of air during filling.

During operation, the vertical position of the "float" varies directly with the rate of flow. This relationship is essentially linear with the new type "float" and is affected relatively little even with very large changes in viscosity (*cf.* Fig. 2). When connected to the electrical unit, the inductance of the rotameter coil varies with the axial position of the iron cylinder within the coil. The inductance change is measured by an AC bridge circuit, the output of which is amplified and ultimately read on a milliammeter. Optical recording is made by reflecting a beam of light from a small mirror fastened to the hand of another series connected milliammeter. (See Fig. 3.)

With a given rotameter, a wide range of sensitivities are available by appropriate adjustments of controls on the electrical unit. Because of minimal interference from changes in blood viscosity, periodic calibrations are not necessary. The entire apparatus is sufficiently stable so that one set of calibrations, made at all (11) gain settings (to obtain different sensitivities), may be used in subsequent experiments to translate, with negligible error, meter readings or recorded deflections in terms of blood flow. In actual practice, one or more calibration points are always taken *in vivo* to verify this relationship. Although the rotameter is primarily a mean flow recorder, the cyclic pulses are not completely damped out and heart rates may be subsequently determined from the photographic record.

Summary. A rotameter and electrical unit have been described with which permanent recordings of blood flow may be made in acute experiments. No special training or technical knowledge is required for its use. Continuous recording of blood flow is possible without any attention from the operator. Tests have shown that the instrument measures blood flow accurately with 5% or less variation and is not significantly altered by changes in blood viscosity or flow pattern over a wide physiological range.

14611

Blood Typing Simplified.

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A method is presented which makes the typing of blood of an individual a very simple, quick and exact procedure. It has been used in 324 cases, resulting in an erroneous result, as judged by older procedures, in only one case.

The necessary equipment is: (1) a blood lancet; (2) two tuberculin syringes with hypodermic needles; (3) toothpicks; (4) alcohol and dry sponges; (5) potent A and B sera.

The A and B sera are prepared by sensitizing known A and B individuals by means of intravenous injection of 0.1 cc of Witebsky's A and B substance.*[1] In most individuals so sensitized an appreciable titer of anti-A or anti-B agglutinin will be present 10 to 12 days following injection. When the titers have reached a high value, in our cases 1:512 anti-A and 1:16,384 anti-B, the individuals are bled 500 cc under sterile technique; the serum is separated, complement inactivated, and stored in sterile containers in the icebox or frozen. The sera so obtained are used undiluted in the test to be described.

The right middle and ring fingers of the individual to be typed are cleaned with alcohol and dried. They are then punctured superficially so that a very small drop of blood (1 mm in diameter) can be expressed on the skin of each finger. A small drop (3 mm in diameter) of A serum is placed from a tuberculin syringe on the blood drop on the middle finger and a similar sized drop of B serum is placed on the blood drop on the ring finger. The sera and blood are mixed with the ends of a toothpick. The mixture is then agitated by snapping the tips of the fingers for a minute and the reactions then read under a suitable light. A positive test, *e.g.*, presence of A factor, is indicated by agglutination of the red blood cells to which the anti-A serum has been added.

Three hundred individuals, chosen at random from the population of the Medical School and the V-12 student body of the undergraduate school, were typed according to the above technic. They were then typed by one of the other 3 methods named in Table I. The typing done according to the glass slide method was done by a pharmacist's mate. He also carried out without difficulty some of the tests, using the author's method, under the author's supervision.

From the results shown in Table I, the reliability of the test is obvious, especially when one realizes that some of the individuals typed as belonging to Group A were later found to be type A_2 and one was proven to be type A_3. These individuals of course give weak reactions when typed by the older methods, and consequently are frequently called Group O. The one inconsistent result was in the 22nd typing done by this new method. Thus there have been no errors in the last 300 typings.

The new test is rapid, so that 2 individuals schooled in the technic can type 70 to 75 individuals in an hour. An individual test can be carried out from start to finish in 2 to 3 minutes. Aside from the ease and rapidity with which the test is carried out, one of its chief assets is the fact that it is impossible to get the individual separated from his test. The individual is present and his blood group is on his fingers. It is then merely necessary to record the name and group at one sitting. Thus the test is ideal for the typing of large numbers of individuals and is so simple in technic and equipment required that the typing of one or more individuals can be carried out as an office procedure.

The M and N type of an individual can

* Supplied through the courtesy of Eli Lilly & Co., Indianapolis, Indiana.

[1] Witebsky, Ernest, Klendshoj, Niels C., and McNeil, Critchon, PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 167.

TABLE I.

Method used	Individuals tested	Errors	% errors	Time required per 25 tests
4-tube	39	0	0	2 hr (estimated)
Hollow-ground slide	36	0	0	45 min (measured)
Glass slide	249	11	4.4	1 hr "
Author's	324	1	0.3	20 min "

All tests done by the author's method were checked by one of the other 3 tests, usually the glass slide method. Whenever there was disagreement between the author's method and the glass slide method the blood was retyped by one of the other 2 methods.

be as simply and quickly determined, carrying out the test as described but substituting anti-M and anti-N sera for anti-A and B sera. Thus on 4 fingers of an individual one can determine in 3 minutes or less his A, B, M, and N types. We have not been successful in using this test to determine the presence or absence of the Rh factor.

I am indebted to Lieut. George P. Heckel, M.C., U.S.N.R., for enabling us to carry out the method on the V-12 students under his care and to Miss Dorothy Miller for her interested cooperation.

Summary. A simple, rapid blood-typing test is described which requires a minimum of equipment, gives clear cut reactions and a very low percentage of error. It is especially useful for typing large groups because of the impossibility of mislabelling the bloods. It is so simple that blood typing can be made a home or office procedure.

14612

A Granular Body Characteristic of Certain Non-Bacterial Pneumonias of Mice.*

J. FURTH AND P. F. DEGARA.

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Several strains of non-bacterial pneumonia readily transmissible by intranasal infection have been established in mice with throat washings or lung suspension from human atypical pneumonias.[1] The lung lesions in 3 of these strains are characterized by large spherical or oval bodies measuring approximately 12 to 30 micra in greatest diameter and composed of myriads of minute granules or short rods (Fig. 1-5). These structures will be designated as granular bodies, and their minute components as elementary particles.

The shape of the granular bodies is sharply defined by a thin capsule or membrane.

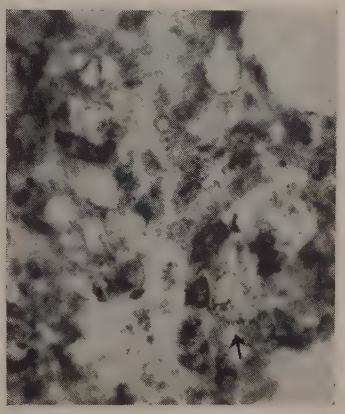

FIG. 1.

Mouse killed 5 days after infection with "mouse pneumonitis virus" (Nigg). The arrows point to 2 characteristic granular bodies. Others in the field are not well in focus. × 400.

* These investigations were made under the Commission on Pneumonia, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the United States Army.

[1] deGara, P. F., and Furth, J., to be published.

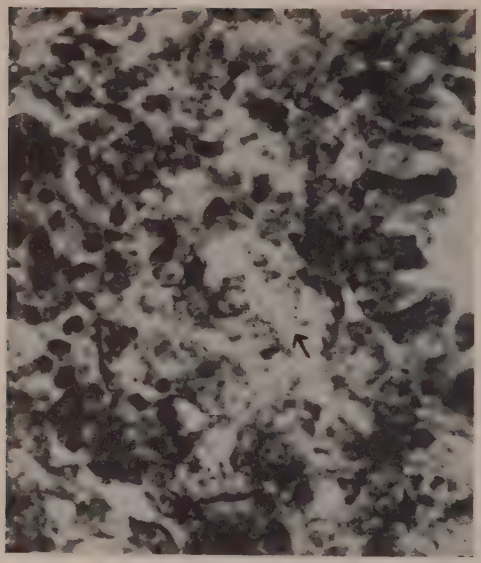

FIG. 2.
Same mouse. Arrow points to a large granular body, part of which is not in focus. × 400.

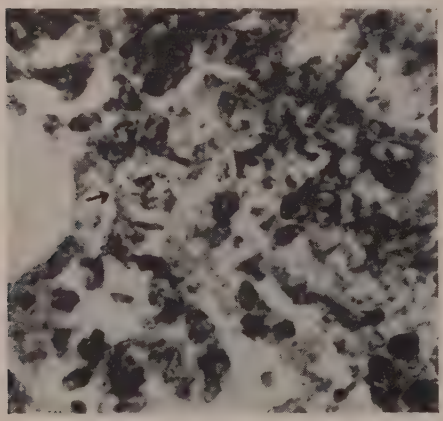

FIG. 3.
Same mouse. Arrow points to granular body, at the margin of which is a spindle-shaped mass, possibly a compressed nucleus. × 400.

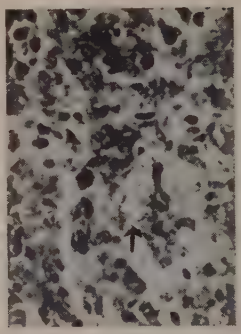

FIG. 4.
Mouse killed 7 days after infection with strain Br62. × 300.

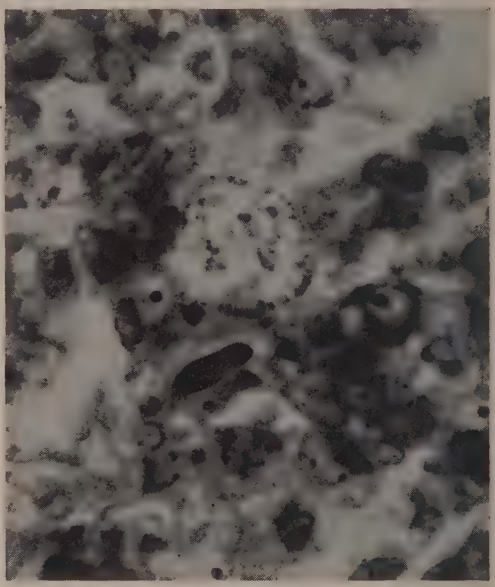

FIG. 5.
Same mouse. The granules are slightly blurred, not being well in focus. × 1300.

Within this membrane are large numbers of minute, spherical elementary particles of more or less uniform size, separated by ample clear space. A few of the particles, notably those close to the membrane, are of the size of a staphylococcus, but most of them are much smaller. In hematoxylin and eosin or Gram-stained sections, the granules are indistinct or unstained, and the lesion resembles a giant vacuole. In Gram-stained preparations the elementary particles are either pale-staining, gram-negative, or unstained. They are well defined in Giemsa-stained sections of tissues fixed in Zenker-formol and embedded in paraffin. Granular bodies were found only in consolidated areas, and were not seen within vessels or in tissues other than the lung.

Virus pneumonia in which these granular

bodies are present is essentially an interstitial inflammation with infiltration of large mononuclear cells and few polymorphonuclear leukocytes. It is accompanied, however, by the accumulation of large numbers of polymorphonuclear leukocytes within the alveoli, with relatively few large mononuclear cells at the onset of consolidation. Minute granules with the size, shape, and staining properties of elementary particles are seen in the cytoplasm of mononuclear cells but seldom in those of polymorphonuclear leukocytes. Inclusions resembling the granular body are rarely seen in the cytoplasm of bronchiolar epithelial cells. These, however, contain numerous coarser particles, and the nucleus of the cell is clearly seen close to the basement membrane, whereas the characteristic granular body is usually devoid of any recognizable cellular component.

The precise relation of the granular body to other structures of the pneumonic lung has thus far not been ascertained. The first impression gained is that the granular bodies represent giant nuclei which have disintegrated into minute particles, but pyknosis and karyorrhexis are not associated with such an enormous swelling of nuclei or with their disintegration into particles so small and uniform. A large and characteristic granular body contains nothing but minute elementary particles of uniform size (Fig. 1, 2). Occasionally a flattened structure, perhaps a nucleus, is seen on the margin of the body (Fig. 3), giving the impression that the granular bodies may have originated from intracytoplasmic accumulation of elementary particles. Very rarely a pale-staining structure, resembling a fading nucleus, is seen within such a body. More common is the presence of larger coccoid particles, the significance of which is likewise uncertain. The granular bodies can be readily distinguished from large mononuclear leukocytes which have phagocytosed cellular debris.

The granular bodies were discovered in the course of attempts to identify the non-bacterial transmissible strain of pneumonia Br62.† In order to ascertain whether their presence is of diagnostic value, pneumonia was produced by different agents, and the lungs, sectioned under comparable conditions, were examined for granular bodies. These were not seen (a) in more than 20 pneumonias produced by influenza virus B,‡ (b) in 10 pneumonias produced by the PVM virus of Horsfall and Hahn§[2] (c) in 5 pneumonias produced by the MP virus of Francis and Magill,‡[3] and (d) in numerous samples of spontaneous pneumonias of mice. In the lesions produced by "mouse pneumonitis virus" of Nigg,§[4] on the contrary, granular bodies were seen in large numbers. Thus, this lesion can be regarded as characteristic of non-bacterial pneumonias of the type produced by strains Nigg[4] and Br.[1] The relation of these two strains to each other and to viruses of lymphogranuloma and psittacosis is under investigation.

Granular bodies were noted as early as 2 days and as late as 21 days after infection of the animal. Strain Br62 is less virulent for mice than the strain of Nigg, and the pneumonias produced by it are slow to develop. After 21 days, however, they may produce as many granular bodies as the Nigg strain after 3 days. The finding of granular bodies proved to be of value in the course of investigations of the non-bacterial pneumonias; *e.g.*, it indicates that the pneumonia was produced by the agent studied and was not an intercurrent disease. In the light of well-known investigations of others (*cf.* [8]) on related elementary particles, this finding strongly suggests that the lesion is still active. In older lesions in which lymphocytes and fibroblast-like cells are numerous in interstitial locations and acute exudate is absent, granular bodies were usually not seen.

† The abbreviation "Br" represents "Fort Bragg," and the number is that of the patient. The material used for inoculation was obtained through the courtesy of Drs. J. H. Dingle and T. J. Abernethy. The relation of the viruses here described to the human pneumonias is under investigation.

‡ These strains were received through the courtesy of Dr. T. P. Magill.

§ These strains were received through the courtesy of Dr. F. L. Horsfall, Jr.

[2] Horsfall, F. L., Jr., and Hahn, R. G., *J. Exp. Med.*, 1940, **71**, 391.

[3] Francis, T., Jr., and Magill, T. P., *J. Exp. Med.*, 1938, **68**, 147.

[4] Nigg, C., *Science*, 1942, **95**, 49.

Granular bodies were not seen in imprints, probably because of their fragility. The current custom is to depend on imprints or smears, which show elementary particles but not granular bodies. This may explain why the granular body has not been noted previously.

In a recent description of lesions caused by the Nigg strain,[5] the presence of intracytoplasmic elementary particles is emphasized, this and other findings linking its agent to those of lymphogranuloma venereum and psittacosis.[5] The intracytoplasmic collection of elementary particles in experimental lymphogranuloma venereum has been named by Miyagawa and associates[6] "granulocorpuscle," and their work has been confirmed by Shaffer, Rake, and McKee.[7] In the yolk sac infected with lymphogranuloma virus, Rake and Jones[8] noted the formation of a "vesicle" containing minute spheroid particles, the whole being similar to the granular body noted by us in the lungs of mice.

Summary. A granular body composed of large numbers of minute spherical particles of uniform size is described in pneumonic consolidations. This lesion appears to be characteristic of certain transmissible non-bacterial pneumonias of mice.

[5] Nigg, C., and Eaton, M. D., *J. Exp. Med.*, 1944, **79**, 477.

[6] Miyagawa, Y., Mitamura, T., Yaoi, H., Ishii, N., Nakajima, H., Okanishi, J., Watanabe, S., and Sato, K., *Jap. J. Exp. Med.*, 1935, **13**, 1.

[7] Shaffer, M. F., Rake, G., and McKee, C. M., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 408.

[8] Rake, G., and Jones, H. P., *J. Exp. Med.*, 1942, **75**, 323.

14613 P

Specificity of Cutaneous Allergy to Procaine in Man.

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This study was carried out on a 26-year-old dentist who was found to respond with itching, vivid erythema, edema, vesiculation, and oozing to contact with aqueous solutions of procaine hydrochloride. The threshold concentration at which this eczematous reaction was just visible, was found to be at 1:36,000 with aqueous solutions of procaine salts (hydrochloride, borate, butyrate), as well as with the base procaine dissolved in triethanolamine.

The cutaneous reactions to procaine and related compounds were tested by the simple drop method, which consisted of applying drops of equal size to the intact skin, and allowing them to remain there for exactly 5 minutes. The reactions were observed and noted 24 hours later. The standard test concentration of all solutions tested was 2½%, *i.e.*, 900 times the threshold concentration of procaine. Bases were dissolved in triethanolamine; salts in water.

Procaine has the structure of an ester:

The two compounds forming this ester, namely, the acid, *p*-aminobenzoic acid, and the alcohol, β-diethyl-amino-ethanol: $HOCH_2CH_2N(C_2H_5)_2$, when tested separately, yielded completely negative skin reactions in our subject. Similarly, seven alkyl esters of *p*-aminobenzoic acid up to C_5, including the isopropyl and isobutyl esters, proved to be without any effect when dissolved in triethanolamine and tested in 2½% concentration. Several aliphatic compounds containing a tertiary amine-N, as does the side chain of procaine, such as triethylamine, $(C_2H_5)_3N$, and its hydrobromide, triethanolamine, $(HOCH_2CH_2)_3N$, and β-chloro-ethyl-diethyl-

* Member of the Staff, Walter G. Zoller Memorial Dental Clinic.

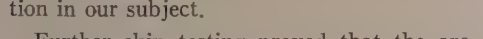
amine, $ClCH_2CH_2N(C_2H_5)_2$, were also ineffective.

The importance of the aromatic amino group for the reaction in question was demonstrated by testing 7 local anesthetics, apothesine, stovaine, alypin, metycaine, diothane, nupercaine, and intracaine, all having a tertiary amine in their side chains, but lacking the aromatic amino group on the benzene ring. None of these compounds caused a skin reaction in our subject.

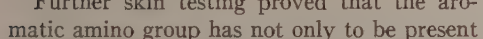
Further skin testing proved that the aromatic amino group has not only to be present on the ring but also must be present in the para-position. We synthesized the diethylamino-ethanol esters of ortho- and meta-aminobenzoic acids, compounds which were different from procaine only in the position of the aromatic amino group. The subject's skin did not respond to these compounds.

Positive reactions, to the same degree as elicited by procaine, were obtained with five compounds, all commercially available local anesthetics:

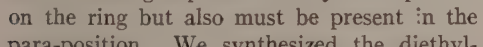
"Pontocaine":



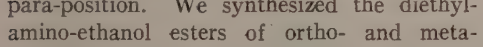
"Monocaine":



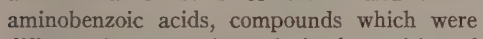
"Larocaine":



"Tutocaine":



"Butyn":



Pontocaine has a side chain similar to that of procaine inasmuch as there are 2 C atoms between the tertiary amine-N and the C atom of the carboxylic group. But it differs from procaine by having an H atom of the aromatic amino group substituted by a butyl radical, and having 2 methyl groups instead of 2 ethyl groups attached to the tertiary amine-N. In monocaine the position of the amine-N is similar to that in procaine and pontocaine but it is a secondary amine, and the attached alkyl chain is different. In the remaining 3 compounds the carboxylic group is separated from the tertiary amine-N by 3 instead of 2 C atoms, and the length of the alkyl chain shows great variations.

Benzoic acid, anilin, *p*-aminophenol, *p*-phenylendiamine and *p*-amino-*m*-hydroxybenzoic-acid-methylester yielded negative results.

The passive transfer test of Prausnitz-Kuestner was negative, an indication that there were no antibodies circulating in the blood.

Discussion. Earlier attempts to establish the range of specificity in epidermal hypersensitiveness to procaine[1,2,3,4] yielded incomplete or contradictory results. In the subject of the present study, the allergic response has required the presence of a *p*-aminobenzoic acid ester, in which the side chain contains a secondary or tertiary amine-nitrogen. The length of this chain may be different. Substitutions of H atoms by alkyl groups in the aromatic amino group or in the side chain have not altered the antigenic effect.

The paramount importance of the position of benzene ring substitutions for the specificity of serological reactions was demonstrated by Landsteiner.[5] Pauling and co-workers[6] showed that para-substituted compounds have greater antigenic effectiveness than compounds with meta- and ortho-substitutions. It seems that the pattern of group specificity in purely epidermal hypersensitiveness, without antibodies in the serum, follows a pattern similar to that of serological reactions.

Summary. In the subject studied eczematous contact type reactions were elicited with a group of *p*-aminobenzoic acid esters which contain a secondary or a tertiary amine in the side chain. The length of the side chain was irrelevant. Substitutions of H atoms by alkyl groups in the aromatic amino group or in the side chain did not alter the antigenic effectiveness. Benzoic acid esters

[1] Schwarzschild, L., *Arch. f. Dermat. und Syph.*, 1928, **156**, 432.

[2] James, B. M., *J. Am. Med. Assn.*, 1931, **97**, 440.

[3] Waldron, G. W., *Proc. Staff Meet. Mayo Clinic*, 1934, **9**, 254.

[4] Goodman, M. H., *J. Invest. Dermat.*, 1939, **2**, 53.

[5] Landsteiner, K., *The Specificity of Serological Reactions*, C. C. Thomas, Springfield, Ill., 1936.

[6] Pressman, D., Brown, D. H., and Pauling, L., *J. Am. Chem. Soc.*, 1942, **64**, 3015.

with a tertiary amine in the side chain but without an aromatic amino group, or with an amino group in meta- or ortho-position, were ineffective.

14614

Evidence that Testicular Androgen Stimulates the Inguinal Bursa of the Adult Rat.*

L. J. WELLS AND M. D. OVERHOLSER.

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In regard to factors which influence the descent of the testis, two recent and related suggestions have been made: (1) that androgen promotes descent by causing the inguinal bursa of Klaatsch[1] to become large enough to receive the testis and (2) that the gubernaculum acting as a mechanical factor assists androgen in expanding the bursa.[2,3] These suggestions have grown out of certain observations upon man[4,5] and rodents. In the ground squirrel, it was found that the gubernaculum is not requisite for the final stages of descent[2] and that injections of androgen increase the weight of the bursa.[3] From earlier investigations it had become clear that in both ground squirrel[6] and rat[7] the weight of the bursa is influenced by 2 synergistic factors: (1) the hormonal action of testicular androgen and (2) the mechanical action of the contents of the bursa. The purpose of the present account is to report in greater detail the earlier investigation in the rat which has been presented hitherto only in the form of an abstract.[7] Recently, Almquist and Andrews have noted that injections of testosterone propionate increase the weight of the bursa and have given us permission to cite their forthcoming paper.[8]

Concerning materials and methods, 29 of 42 rats were castrated (Tables I and II). A median abdominal incision was made and castration was performed either bilaterally (25 cases) or unilaterally (4 cases). In all instances except 7, the gubernaculum and the mesentery, supporting epididymis and ductus deferens, were severed, then the spermatic vessels were ligated in the lumbar region, and the testis removed together with the epididymis, the greater part of the ductus deferens and most of the "lumbar fat body." In the 7 exceptional instances, no structure except the testis was removed from the left side of the animal; after the spermatic vessels had been ligated at a point near the mediastinum testis and then severed, the testis was removed by shelling it from the epididymis. Generally the orifice of the bursa was permitted to remain open. However, in each of 8 rats, a sterile pellet of paraffin was placed in the right bursa and the orifice was closed by sutures. At autopsy, the various bursæ were removed, weighed to the nearest tenth of a milligram and placed in Bouin's solution. Twenty-two of the bursæ were sectioned at 7 μ and the sections were stained with hematoxylin

* Aided by a grant from the medical research funds of the Graduate School of the University of Minnesota and a grant from the Research Council of the University of Missouri.

1 Klaatsch, H., *Morph. Jahrb.*, 1890, **16**, 587.

2 Wells, L. J., *Anat. Rec.*, 1944, **88**, 465 (Suppl. 4, April).

3 Wells, L. J., *Anat. Rec.*, 1944, **88**, 465 (Suppl. 4, April).

4 Wells, L. J., *Surgery*, 1943, **14**, 436.

5 Wells, L. J., *Bull. Minnesota Med. Found.*, 1944, **4**, 50.

6 Wells, L. J., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 625.

7 Wells, L. J., and Overholser, M. D., *Anat. Rec.*, 1937, **70**, 121 (Suppl. 1, December).

8 Almquist, J. O., and Andrews, F. N., *Anat. Rec.*, 1944, **89** (June, in press).

TABLE I.
Unoperated Controls.

Rat			Autopsy			
				Weight of organs in mg		
Litter	No.	Age at autopsy, days	Body wt, g	Testes	Seminal vesicles	Bursæ Right	Bursæ Left
A	1	130	324	3095	729*	427	449
B	2	134	342	2830	1172	498	450
C	3	140	334	3030	1141	424	381
D	4	170	360	3106	1398	485	419
E	5	170	320	2926	1071	344	347
F	6	174	278	2641	657*	351	342
G	7	179	270	2633	1063	317	313
H	8	182	379	3123	1144	528	482
I	9	321	285	2304	310	372	348
Lot J†	10	Ca. 4 mo.	308	2965	1206	435	458
	11	" 4 "	294	2792	1113	365	358
Lot K†	12	" 11 "	295	2268	357	353	335
	13	" 11 "	252	2115	198	326	312

* Before weighing the seminal vesicles, an appreciable quantity of their stored secretion was accidentally lost.

† In each of these lots, data from the 2 rats were averaged and then used as controls.

and eosin (bursæ from Rats 3, 4, 14, 16, 18, 19, 20, 23, 25, 26, 27, 36, 37, 41 and 42). In calculating the percentages in Table II, littermate controls were used. For example, Rat 1 (Table I) was the littermate control for Rats 14, 15, and 16.

Three of the unoperated controls had become so senile that at autopsy their seminal vesicles already had undergone regression (Rats 9, 12 and 13). That this regression tended to minimize the relative effects of bilateral castration on the seminal vesicles is shown in Table II. In spite of this tendency, however, it does not necessarily follow that in testing the effects of bilateral castration on the bursa the most convincing data should be those derived from the younger rats (those which were killed on or before the 182nd day of life). While castration deprives seminal vesicles of androgen alone, it deprives bursæ of both androgen and contents. Furthermore, data from the 13 controls show that the weights of right and left bursæ tend to exhibit a closer relation to weight of testes than to weight of seminal vesicles.

Bursa deprived of androgen and of contents. From the percentages in Table II, it may be judged that in most instances bilateral castration induced a remarkable decrease in the weight of the bursa. When the percentages of the 35 empty bursæ were arranged according to magnitude (those bursæ not marked by GE or Lf), it was found that the range was from 18 to 74 and that the median was 47. In regard to the larger percentages (70, 73, and 74), perhaps their magnitude was influenced by the inflammation of certain bursæ. That the right bursæ of Rats 37, 41, and 42 had been inflamed is suggested by the fact that their lumina exhibited several polymorphonuclear leucocytes.

Bursa deprived of androgen and part of contents. In each of 8 rats (17, 21, 26, 29, 30, 40, 41, and 42), one bursa was empty and the other bursa contained either lumbar fat (Lf) or gubernaculum and epididymis (GE). Note that in each of these rats except No. 40 the percentage weight of the empty bursa was smaller than that of the other. Likewise, observe that except in Rat 40 the absolute weight of the empty bursa was smaller than that of the contralateral bursa.

Right bursa deprived of contents. Two of the rats which were unilaterally castrated are favorable animals for observing the effects of depriving one bursa of its contents but not of androgen (Rats 15 and 24). In each of these 2 rats, the left bursa served as control for the

TABLE II.
Effects of Castration on Weight of Bursa.

Castrated rat			Autopsy						
				Weight of organs					
						Bursæ			
				Seminal vesicles		Right		Left	
Litter	No.	Age at autopsy, days	Days after operation	Mg	% of control	Mg	% of control	Mg	% of control
A*	14 P	130	51	63	9	—	—	153	34
	15 Cu	130	51	872	119	262	61	412	—
	16 Cu,P	130	51	937	128	—	—	445	—
B	17	134	32	85	8	134	27	150	33GE
C	18 P	140	51	56	5	—	—	127	33
	19 P	140	51	53	5	—	—	132	34
D	20 P	170	51	73	5	—	—	124	21
E	21	170	51	68	7	151	44	174	50GE
F*	22	174	51	78	9	168	48	168	49
	23 P	174	51	80	10	—	—	137	40
	24 Cu	174	51	810	122	289	82	347	—
	25 Cu,P	174	51	768	117	—	—	367	—
G	26	179	51	70	7	200	63Lf	173	55
H	27 P	182	51	89	8	—	—	86	18
I	28	321	51	101	32	101	27	—	—
	29	321	51	82	26	115	31	132	38GE
	30	321	51	105	34	176	47	217	62GE
Lot J	31	Ca. 4 mo.	32	93	8	173	43	152	37
	32	" 4 "	32	85	8	178	45	189	47
	33	" 4 "	32	94	8	198	49	177	44
	34	" 4 "	32	130	11	179	45	182	45
Lot K	35	" 11 "	33	92	31	214	63	216	66
	36	" 11 "	33	104	34	165	49	146	58
	37	" 11 "	33	108	36	233	70	186	58
	38	" 11 "	33	111	37	212	62	194	60
	39	" 11 "	33	147	49	186	55	187	58
	40	" 11 "	33	88	27	180	53	173	53GE
	41	" 11 "	33	110	36	249	73	255	79GE
	42	" 11 "	33	123	41	253	74	284	84GE

* Before weighing the seminal vesicles of the unoperated control, some of the stored secretion was accidentally lost.

Cu—Castrated unilaterally (right bursa emptied).

GE—Left gubernaculum and epididymis not surgically removed.

Lf—Lumbar fat occupied the right bursa at autopsy. It was attached to the peritoneal layer of the bursa by an adhesion.

P—Paraffin pellet in right bursa (see text).

right; the absolute weight of the empty bursa was smaller than that of the control. The fact that the left testis secreted sufficient androgen is shown by the percentage weight of the seminal vesicles.

Bursæ containing paraffin. When sections of the 8 bursæ which contained paraffin were studied microscopically, it was found that each bursa was lined by a thick shell of connective tissue that undoubtedly had originated as a consequence of chronic inflammation. Since this shell was so thick (148 to 825 μ) that it

must have contributed heavily to the weight of the bursa, the weights of the 8 bursæ containing paraffin do not constitute acceptable data concerning the influence of mechanical distention upon the weight of the bursa; therefore these data are not presented in Table II. Furthermore, in the remarks which follow, only data other than these are considered.

Discussion and conclusion. Regression of the bursa induced by unilateral castration (mechanical factor) was less than that induced by bilateral castration (hormonal and mechanical factors); compare Rats 14 and 15 and Rats 22, 23, and 24. In 6 of 7 rats which had been bilaterally castrated, the data suggest that regression of the bursa was hindered by the presence of such structures as the epididymis (mechanical factor). It is concluded that in the adult rat the weight of the bursa is influenced by two synergistic factors: (1) the hormonal action of testicular androgen and (2) the mechanical action of the contents of the bursa.

14615

Acute Anatomic Breakdown of Motor End Plates in Hemorrhagic Shock.*

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The pathology of the motor end plates in voluntary muscle during hemorrhagic shock is unknown. The effects of strong electric and chemical shock,[1] poliomyelitis,[2], pneumonia, other acute infections,[3] and lactic acid,[4] in producing either a local or general loss of structural and functional innervation at the myoneural junctions, have been demonstrated. Shock caused by hemorrhage,[5] trauma,[6] or gravity,[7] produces a state of anoxia followed by an accumulation of lactic acid (hyperlactacidemia) and keto acids (pyruvic, acetoacetic, etc.) in the blood. Accumulation of lactic acid increases the neurohumoral stimulant by retarding the destruction of physiologically deposited acetylcholine at the synapses.[8] The claim is made that there are no definite grounds for differentiating between hemorrhagic shock and shock from other causes.[9] The structural alterations of the neuromuscular apparatus during shock from any cause, however, remain an unexplored field.

Methods. Thirty albino rats weighing from 150 to 250 g were used. All experiments were performed after anesthesia with sodium pentobarbital (nembutal), 4 mg per 100 g intraperitoneally injected. Shock was induced by bleeding from the cut tails according to the method of Engel, Winton, and Long.[10] Blood was removed, over a period of one hour,

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

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9 Weston, R. E., Janota, M., Levinson, S. O., and Necheles, H., *Am. J. Physiol.*, 1943, **138**, 450.

10 Engel, F. L., Winton, M. G., and Long, C. N. H., *J. Exp. Med.*, 1943, **77**, 397.

equivalent to 3 to 4% of the body weight. After shock had been established no further anesthesia was necessary. It was impossible to produce shock in all rats by the withdrawal of a standard amount of blood over a one-hour period because of individual differences inherent in each rat. The clinical criteria for shock were pallor, cyanosis, cold extremities, tachypnea, sluggish or absent blood flow from cut tail, increased salivation and lacrimation, and, after 6 to 12 hours, so-called bloody tears or "chromodacryorrhea" in those animals in irreversible shock. There was muscle weakness, hypotonia, atonia, and flaccid paralysis of certain voluntary muscles, in some cases preceded by coarse tremors. In some animals certain muscles were in a state of antemortem rigidity and failed to respond to either direct or indirect electrical or mechanical stimulation. Fourteen of the 30 animals died in profound shock within 24 hours. Five normal control animals were maintained under nembutal and 5 under ether anesthesia for the same periods as were the bled rats. The following muscles, either of the right or of the left side, were removed from each of 5 living rats at 2- and 4-hour intervals after bleeding began: sternomastoid, triceps, intercostals, rectus abdominis, rectus femoris, gastrocnemius, quadriceps femoris. These muscles were processed at once, by many methods, especially the gold technic, and later teased.[11] Over 1200 teased preparations were used in this study. The muscles from rats that survived the first 24-hour period were not used in this study. Blood pressures were determined in several instances by direct cannulation of the carotid artery, heparin being used as an anticoagulant. The pressure was read on a mercury manometer. Heparin in saline was used in the system.

Results. The normal structural variations in the motor end plates of the sternomastoid and intercostal muscles were established previously.[2-4] During the first 2 hours after bleeding began, the majority of the end plates were retracted into globoid masses surrounded by Kühne's granules; both the masses and the granules took an intense impregnation with gold. The epilemmal axon was beaded and at its junction with the hypolemmal axon there was either a fine filament or a large droplet of the axon. When the connection between epilemmal and hypolemmal axons was a fine filament, the center of the end plate was open and decreased in gold impregnation. When the end plate in full surface view had a large dark droplet at the connecting link with the epilemmal axon there was a wheel appearance with a large dark hub formed by the axonic droplet.

From 2 to 6 hours after bleeding began, the majority of the end plates were expanded and composed of fine ramifications depleted of Kühne's granules. Those animals in irreversible shock for 6 to 24 hours had a progressive granulation leading to liquefaction of the majority of the motor end plates. In different end plates of a single motor tree, the large gold staining droplets of the epilemmal axons could be traced in different locations progressing through the hypolemmal axons of the end plate and then projected out into the related muscle fibers.

Hemorrhagic shock developed conditions in which, instead of the fine granules of Kühne, large masses of axonic substance penetrated the end plate because of the increased permeability. These axonic materials in muscle formed either large amorphous masses darkly impregnated with gold and measuring from 10 to 200 microns in length and from 5 to 25 microns in width, or small oval or fusiform bodies that had a definitely cross striated structure which did or did not agree with the periodicity of the muscle fiber. These axonic masses projected into the muscle fiber and disconnected from the motor tree were comparable to what was previously described as "inclusion masses" found in the muscles of monkeys during the early stages of experimental poliomyelitis (*cf.* Figs. 3 and 4).[2] Coincident in time with the projection of these masses from the hypolemmal axon out into the muscle fiber, there was a corresponding depletion or exhaustion of the epilemmal and hypolemmal motor axons resulting in loss of innervation of the voluntary muscle. During the end stage of shock, 5458 end plates were denuded from 6000 epilemmal axons counted

[11] Carey, E. J., *Anat. Rec.*, 1941, **81**, 393.

in the gastrocnemius muscle from a rat that had been in profound shock for 14 hours. This is comparable to the denudation of end plates by experimental poliomyelitis (*cf.* Figs. 5 and 6)[2] and injection of lactic acid locally in the zone of motor innervation of skeletal muscle (*cf.* Figs. 2 to 5).[4] The number of epilemmal axons denuded of end plates or possessing pathologic end plates varied in different fibers within a single muscle as well as in different muscles of the body. The intercostal muscles from the rat in shock for 14 hours were characterized in many places by large masses of axonic material that had flowed out from the end plates into the muscle fibers as well as between them. This axonic substance appeared to have drained right through the terminals of the motor nerves and to have left them in a depleted condition. In the final stage of irreversible shock, stimulation of the sciatic nerve failed in many instances to cause a response in the gastrocnemius and certain other muscles. Many muscles in the end stage of shock were in a greatly reduced state of irritability or had lost completely the capacity to respond to direct electric or mechanical stimulation. Many muscle fibers whose irritability was reduced or lost had so-called Zenker's waxy and granular degeneration with irregularities and loss of cross striations, increased visibility of pycnotic nuclei, and perivascular infiltration of leucocytes. Some of the intramuscular capillaries and venules were dilated and contained agglutinations of red blood cells. In other locations these vessels were collapsed and devoid of cellular constituents. Prolonged anesthesia with nembutal produced retraction and accumulation of Kühne's granules of the majority of the end plates, whereas ether expanded many of the end plates depleted of Kühne's granules. The changes produced by anesthesia are not as pronounced as those produced by irreversible hemorrhagic shock. In shock there is, in addition to those changes in the end plates produced by anesthesia, complete granulation, liquefaction, and projection into the muscle of axonic material, of many of the motor end plates. This produces loss of structure and function of the voluntary motor innervation at the neuromuscular junction.

Comments. "Chromodacryorrhea" or the shedding of so-called bloody tears, is produced in rats by injecting dacryorrhetin, a compound prepared from muscle,[12] and by acetylcholine.[13] Acetylcholine when injected into rats brings about an excessive secretion of saliva, intense lacrimation, and the appearance of a material in the tears which greatly resembles blood. This material, an excretory product of the Harderian gland, has been identified spectroscopically as a mixture of protoporphyrin and coproporphyrin. Tashiro proposed that the excretion to which he applied the name "chromodacryorrhea" be used as a biological assay for acetylcholine. A study has been made on the removal of acetylcholine by cholinesterase injections and the effect thereof on nerve impulse transmission[14] to the muscles of the iris. The relationship of dacryorrhetin[12] to adenosine triphosphate[15] is unknown. If the so-called bloody tears were produced by acetylcholine the phenomenon should have appeared early in shock. The fact that the bloody tears appeared in the terminal stage of shock suggests that some breakdown product accumulates in the blood stream during anerobic muscle metabolism. Additional evidence is needed to clear this point. The nervous exhaustion theory,[16,17] prevalent during the Civil War period, recently has been revived.[18–21] Possibly when the pathologic

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[13] Freud, J., *Acta Brevia Neerl.*, 1933, **3**, 159; Selye, H., *Canad. Med. Assn. J.*, 1937, **36**, 200; Tashiro, S., *Kongressbericht. II des XVI Internat. Physiologenkongress*, 1938, 46.

[14] Mendel, B., and Hawkins, R. D., *J. Neurophysiol.*, 1943, **6**, 431.

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[16] Michell, S. W., *New York Med. J.*, 1866, **2**, 321.

[17] Gross, S. D., *System of Surgery*, Phila., Lea, 1872, v. I, p. 426.

[18] O'Shaughnessy, L., and Slome, D., *Brit. J. Surg.*, 1935, **22**, 589.

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mechanism of shock is completely known the voluntary neuromuscular factor may be found closely related to the production of toxins[22-25] and to local fluid loss from capillaries.[26-28]

Summary. The limited evidence in this paper supports the claim that hemorrhagic shock profoundly alters the morphology of the motor end plates and finally produces loss of structural innervation of many muscle fibers in a single voluntary muscle. This histologic change is highly irregular in the different muscles of the rat, therefore large numbers of specimens from different muscles were teased. Gold staining masses of axonic materials drain out into and between the muscle fibers coincident in time with the loss of motor innervation due to the increased permeability of the end plates. The epilemmal axons, exhausted of their substances, are in many places likewise denuded of their hypolemmal end plates. There is therefore a real anatomic breakdown of many motor end plates and histologic alteration of certain skeletal muscles in hemorrhagic shock.

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Cutaneous Application of Ethinyl Estradiol in Alcohol.

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It has been shown by Zondek[1] and Moore[2] that the estrogens, estrone and estradiol benzoate, respectively, were absorbed through the skin when administered by inunction in any oily vehicle. Later Zondek[3] showed that percutaneous absorption was even more effective when estrone was placed in an alcoholic solvent.

With the introduction of ethinyl estradiol, the new synthetic ester of the natural steroid estrogen estradiol in which the hydroxyl of carbon 17 is replaced with an ethine group,[4] it was considered advisable to test for percutaneous absorption of this hormone, in an alcoholic solution, on the pituitary, gonads, reproductive tract and body growth of the albino rat.

Experimental procedure and methods. Ethinyl estradiol‡ was dissolved in absolute alcohol to facilitate evaporation after application. One calibrated drop, or 2 in a few

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4 Inhoffen, H. H., and Hohlweg, H., *Naturwissenschaften*, 1938, **6**, 96.

‡ Grateful acknowledgment is made to Dr. Gregory Stragnell and the Schering Corporation for the ethinyl estradiol.

TABLE I.
Sample Data of Effects of Ethinyl Estradiol on Adult White Rats (20 daily treatments).

No. of animals	Avg body wt gain (g)	Daily dosage (γ)	Avg wt compared to normal†		
			(2) Ovaries and fallopian tubes	Uterus	Pituitary
		Series I—Spayed Adult Females—age, 3½-4 months. (Spayed 21 days prior to treatment; 20 daily treatments; autopsy day 21.)			
4	71	Con.	—	1.00	1.00
2	45	0.10*	—	5.34	2.40
2	54	0.25	—	3.63	2.20
3	23	1.00	—	4.20	1.40
3	36	10.00	—	6.22	4.00
2	24	20.00	—	4.04	2.60
		Series II—Adult Intact Females—age 3½-4 months. (20 daily treatments; autopsy day 21.)			
4	53	Con.	1.00	1.00	1.00
4	38	0.10	0.84	0.92	1.67
3	41	0.25	0.88	1.08	1.50
3	31	1.00	0.79	1.02	1.50
3	26	10.00	1.00	1.43	3.00
3	37	20.00	1.05	1.50	2.17
1	18	40.00	1.28	1.44	2.67

			(2) Testes	(2) Seminal vesicles	Pituitary	Ventral Prostate
		Series III—Castrated Adult Males. (Castrated day 98; treatments days 122-142; autopsy day 143.)				
2	14	Con.	—	1.00	1.00	1.00
2	−4	0.25	—	1.30	1.33	1.60
2	2	1.00	—	1.78	1.67	2.00
1	−4	20.00	—	1.74	2.67	2.00
1	30	40.00	—	1.83	1.83	1.60
		Series IV—Adult Intact Males. (Treatments, days 84-104; autopsy, day 105.)				
2	23	Con.	1.00	1.00	1.00	1.00
2	7	0.25	0.73	0.22	1.25	0.31
1	12	1.00	0.23	0.22	1.00	0.18
1	24	20.00	0.23	0.20	3.00	0.16

* 1 γ = 10 rat units.
† Normal = 1.00, calculated on average weight in mg/100 g of body weight.

cases, of this solution containing a known amount of the crystalline hormone (0.1 γ to 40.0 γ/day, Table I) was applied daily for 20 days without massage, to a clean-shaven area on the suprascapular region of albino rats. Littermates and rats of comparable ages were used and separately caged so as to avoid contamination of the treated area.

Sixty-nine rats were divided into 4 series with suitable controls as follows: Series I (20 animals), spayed females—both immature and adult; Series II (26 animals), intact females—immature and adult; Series III (10 animals), castrated males—immature and adult; and Series IV (13 animals), intact males—immature and adult. Vaginal smears from all females were made daily starting 5 days before the first application. Autopsy was made on the day following the last treatment and fresh weights of tissues obtained. The tissues were then fixed and prepared for histological examination.

To determine whether the effect of ethinyl estradiol on the body weight increase was significant, it was necessary to compute the "P" value of the body weight gains of each treated group of each dosage level and of all the treated animals taken as one group regardless of dosage (Table II). These statistics were calculated by determining the significance of difference between means of small samples and the "P" value obtained by use of "Student's" t-test for unique samples.[5]

Results and Summary. 1. The estrogenic

[5] Fisher, R. A., *Statistical Methods for Research Workers.*

TABLE II.
"P" Values and Body Weight Gains of Treated Groups Compared to Untreated Groups.

$$\text{S.D.} = \sqrt{\text{V}\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}$$

Series	Group	Avg body wt gain (g) Control	Treated	"P" value*
I.	Adult spayed females	71	34	<0.01
II.	" intact "	53	34	0.03
III.	Castrated adult males	14	4	0.60
IV.	Immature intact "	65	36	0.04
"	Adult " "	23	12	0.40

* <0.05 is significant.

hormone, ethinyl estradiol, in an alcoholic solution was absorbed through the skin of albino rats very effectively by cutaneous application without massage and in this vehicle appeared to give a complete substitution for the natural estrogenic internal secretion from the ovary. This would seem to indicate a very simple and reliable means of cutaneous application. Effective absorption of this estrogen through the skin was evidenced by the pronounced influence similar to that generally known of other estrogens on the pituitaries, ovaries, uteri, vagina, testes, seminal vesicles and ventral prostates as determined by gross weights (Table I) and histological studies. 2. The potency of an alcoholic solution of ethinyl estradiol applied to the skin was further substantiated by the fact that estrus was induced in both spayed and intact females in 24 hours and that the daily dosage of 0.1 γ was sufficient to substitute for the loss of the ovaries in castrates as evidenced by uterine weights (Table I). 3. Some retardation of body growth gain (Table II) was caused by the estrogenic treatment in the adult females (spayed and intact) and in immature males. Whether the retardation of body growth was due to some specific toxicity or to some other factor was not determined, however, no visible toxic effects were witnessed in daily observations on the eating habits, general skin and hair condition.

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Effect of Sulfhydryl Reagents on Adenosinetriphosphatase Activity of Myosin.

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Myosin, the main constituent of contractile muscle fibers, has been identified by Engelhardt and Ljubimova[1,2] with adenosinetriphosphatase, the enzyme which catalyzes the breakdown of adenosinetriphosphate (ATP) to adenosinediphosphate and inorganic phosphate. Needham[3] and Bailey[4] have confirmed this discovery.

Needham[3] reported that iodoacetate had no effect on the adenosinetriphosphatase activity of myosin, while glycine and ammonium chloride (reagents known to abolish the nitroprusside test of myosin) actually increased enzyme activity. Needham came to the con-

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[2] Ljubimova, M. N., and Engelhardt, V. A., *Biochimiya*, 1939, **4**, 716.

[3] Needham, D. D., *Biochem. J.*, 1942, **36**, 113.

[4] Bailey, K., *Biochem. J.*, 1942, **36**, 121.

clusion that the -SH groups of myosin were not essential for adenosinetriphosphatase activity.

In a preliminary note[5] on sulfhydryl enzymes data were presented showing that the adenosinetriphosphatase activity of myosin was abolished on addition of *p*-chloromercuribenzoic acid (*p*-ClHg benzoate) and was restored on addition of glutathione (GSH). It was concluded from this inhibition and reactivation that adenosinetriphosphatase was an -SH enzyme.* The -SH groups of myosin have been determined by a number of investigators.[6,7,8] Evidence for the identity of myosin with adenosinetriphosphatase could be obtained by studying the relation between the –SH groups of myosin and enzyme activity in a manner similar to that followed by Hellerman *et al.*[9] in their studies on urease. If the gradual abolition of the –SH groups of myosin by mercaptide forming reagents is followed by a gradual abolition of enzyme activity, this may be considered as a strong argument for the identity of myosin with adenosinetriphosphatase.

Experimental. Myosin was prepared according to Bailey;[4] it had an initial adenosinetriphosphatase activity of Q_P = 1150-1300 in carbonate buffer, pH 8.52 at 38° (Q_P = cmm P liberated per mg dry weight per h.); the activity in 0.1 *M* glycine buffer-0.1 *M* NaCl, pH 8.78 was Q_P = 2350-2400 at 38°. The –SH groups of myosin were estimated by the ferricyanide method of Anson,[10] the iodosobenzoate method of Hellerman *et al.*,[11] and by porphyrindin titration with nitroprusside as an internal indicator.[8] The "freely reacting" sulfhydryl groups (those –SH groups in the *native* protein which give a positive nitroprusside test and react rapidly with mild oxidizing agents), and the total sulfhydryl content of the protein (–SH groups determined in the presence of 1 g guanidine HCl per cc of protein solution) were separately determined. Adenosinetriphosphatase activity was measured by making the enzyme concentration the rate determining step in the reaction: Adenosinetriphosphate ⇌ adenosinediphosphate + PO_4H_3, and estimating the inorganic phosphate liberated.

Results. The freely reacting –SH content of native myosin was 0.33-0.35 micromole –SH per 10 mg protein by both the porphyrindin and the *p*-ClHg benzoate methods. This amount of native myosin reduced 0.34 micromole of ferricyanide. The total –SH content of guanidine HCl-denatured myosin gave 1.0 micromole –SH per 10 mg protein by the porphyrindin method and 1.08 micromoles by the iodosobenzoate method. The values are in good agreement with the values reported by Greenstein and Edsall.[8] Titration of the total –SH content by *p*-ClHg benzoate gave a lower figure: 0.73 micromole per 10 mg (in 10 *M* guanidine HCl). The reason for this discrepancy is not clear.

The evidence that sulfhydryl groups are necessary for adenosinetriphosphatase activity is summarized in Table I. *p*-ClHg benzoate (1.3×10^{-5} *M*) inhibited the enzyme completely, while 3-NH_2, 4-OH phenyldichloroarsine HCl (6×10^{-4} *M*) produced 48% inhibition. Where mercaptide-forming reagents were used, the inhibitions were completely reversed by 10 equivalents of GSH added 20 minutes after the addition of the inhibitor. As a matter of fact, GSH (5×10^{-4} *M*) raised the activity of older preparations 20-40% above the level of the controls, probably by reducing some of the –SH groups that had been oxidized on standing. The inhibition with porphyrindin (1.3×10^{-5} *M*) was 32%. These data are taken as evidence for the sulfhydryl nature of the enzyme, as in our experience organic mercurials and arsenicals are the most reliable reagents for the detection

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* After this paper was submitted for publication, M. Ziff (*J. Biol. Chem.*, 1944, **153**, 25) and J. W. Mehl (*Science*, 1944, **99**, 518) have confirmed our experiments on the reversible inhibition of adenosinetriphosphatase by –SH reagents.

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9 Hellerman, L., Chinard, F. P., and Deitz, V. R., *J. Biol. Chem.*, 1943, **147**, 443.

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11 Hellerman, L., Chinard, F. P., and Ramsdell, P. A., *J. Am. Chem. Soc.*, 1941, **63**, 2551.

TABLE I.
Effect of Sulfhydryl Reagents on Adenosinetriphosphatase Activity of Myosin.

Exp.	Inhibitor	Concentration of inhibitor	Inorganic phosphate liberated (γ P) Control	Inhibitor	Inhibition (%)
1*	3-NH_2, 4-OH phenyldichloroarsine HCl	6x10-4 *M*	25.6	13.1	48
2†	*p*-ClHg benzoate	1.3x10-5 *M*	27.8	0	100
3†	Porphyrindin	1.3x10-5 *M*	27.8	18.8	32
4†	Iodoacetamide	3x10-3 *M*	27.8	27.8	0

* Enzyme, 1.0 cc (≏ 0.17 mg N) in 0.5 *M* KCl—0.05 *M* veronal, pH 7.4. ATP, 0.5 cc (≏ 0.14 mg) 7 min. P (P hydrolyzed in 7 min. in 1 *N* HCl at 100°). $CaCl_2$, 0.05 cc, 0.18 *M*. Temperature, 38°. Duration of experiment, 10 min.

† Same as above, but 1.0 cc enzyme 0.34 mg N; duration of experiment, 5 min.

of –SH-enzymes.

Concerning the failure of inhibition by iodoacetamide, the reason may be either that those groups which alkylating agents attack are not essential for adenosinetriphosphatase activity (this is the case with urease)[11] or that under the experimental conditions iodoacetamide does not combine with any of the –SH groups of the enzyme. When only the freely reacting sulfhydryl groups of myosin were destroyed by stoichiometric amounts of *p*-ClHg benzoate or excess ferricyanide, porphyrindin, or iodosobenzoate,* an inhibition of 11 to 16% occurred (Table II). If iodoacetamide had combined with these reactive –SH groups, this much inhibition would have been evident. Evidence that the freely reacting –SH groups of the protein are not attacked by iodoacetamide comes from the observation that myosin, after treatment with iodoacetamide as in Table II gave a strong nitroprusside test both in the native state and after denaturation with guanidine HCl. Furthermore, myosin treated with iodoacetamide reduced the same amount of ferricyanide as the untreated control. Under these conditions, then, iodoacetamide did not react with the –SH groups of myosin, and if the enzyme were identical with myosin, this would explain the lack of inhibition by alkylating agents.

In order to determine the correlation between abolition of the –SH groups of myosin and the extent of inhibition of adenosinetriphosphatase activity, Hellerman's reagent, *p*-ClHg benzoate, was chosen as the -SH reagent. It was made up in 0.5 *M* KCl in a concentration of 1×10^{-3} *M* by weight. (By titration against standard GSH to nitroprusside end point, this solution was 1.06×10^{-3} *N*). One cc portions of 0.97% solution of myosin were treated with varying amounts of the standard *p*-ClHg benzoate. After 3 minutes the solutions were diluted with veronal buffer (0.05 *M*, pH 7.1, 0.5 *M* in KCl) to 25 cc. One cc aliquots of such solutions were analyzed for adenosinetriphosphatase activity, duplicate assays checking within 2% (Table II). The 1 cc portions of myosin solution contained 1.0 micromole total –SH as determined by porphyrindin titration and 0.28 micromole freely reacting –SH groups as determined by porphyrindin and *p*-ClHg benzoate titrations. Fig. 1 gives the results of a typical experiment. The abscissæ represent the freely reacting and total –SH content of 1 cc of myosin solution as well as the micromoles of *p*-ClHg benzoate added. The ordinate represents the degree of inhibition of enzyme activity. It is apparent that 0.1 micromole of *p*-ClHg benzoate caused no inhibition of enzyme activity although 30% of the freely reacting –SH and 10% of the total –SH groups were abolished. Abolition of all the freely reacting –SH groups (as checked by nitroprusside test) produced 11% inhibition of enzyme activity; as soon as all these –SH groups were destroyed, a steep rise in enzyme inhibition occurred, indicating that the sluggish –SH groups attacked from this point on were more essential for enzyme activity.

If the porphyrindin titer of the total –SH content is chosen as the correct value, 70%

* These oxidizing agents attack only freely reacting –SH groups in the native myosin.

TABLE II.
Relation of Amount of Myosin –SH to Adenosinetriphosphatase Activity.

Reagent	Amt. reagent added to 1 cc of myosin* (micromoles)	Myosin –SH groups destroyed by reagents†		Inhibition of adenosine-triphosphatase activity (%)
		Freely reacting –SH	Total –SH (%)	
p-ClHg benzoate	0.28	all‡	40	11
" "	0.42	all	60	53
" "	0.60	all	86	96
Porphyrindine	0.50§	all	40	14
o-iodosobenzoate	0.50§	all	40	16
Ferricyanide	50.0	all	40	11
Iodoacetamide	1.0	0	0	0

* One cc of 0.97% myosin in 0.5 *M* KCl; freely reacting –SH titer = 0.28 micromoles per cc (by ferricyanide and porphyrindin); total –SH titer = 1.0 micromoles per cc (by porphyrindin and iodosobenzoate); total –SH titer by *p*-ClHg benzoate to nitroprusside end point = 0.70 micromoles per cc. To 1 cc of this myosin the above reagents and then enough 0.05 *M* veronal (pH 7.1, 0.5 *M* in KCl) were added to bring the volume to 25 cc. After 15 minutes a 1 cc aliquot was tested for activity; conditions as in Table I.

† These values are calculated in terms of titrations with the respective –SH reagents. The oxidizing agents in the table react only with the freely reacting –SH groups.

‡ The amount of reagent added is just enough to abolish the nitroprusside test.

§ 0.50 = 1.0 micromoles –SH.

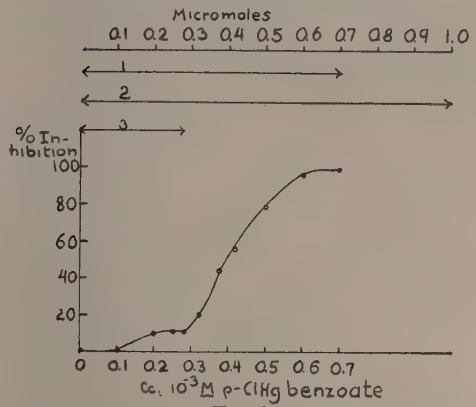

FIG. 1.

Correlation Between the –SH Groups of Myosin and Adenosinetriphosphatase Activity.

Upper abscissæ—

1 = "Total –SH" content by p-ClHg benzoate titration.

2 = "Total –SH" content by porphyrindin titration.

3 = "Freely reactive –SH" content by porphyrindin titration and by reduction of ferricyanide.

Lower abscissæ—

Amounts of ClHg benzoate added to produce gradual destruction of –SH groups of myosin.

Ordinate—

Inhibition of enzyme activity (%).

of the sulfhydryl content of the myosin is destroyed before complete inhibition of enzyme activity occurs; if the *p*-ClHg benzoate titer (to a nitroprusside end point) is selected as the basis of the calculation, then 85-90% of the sulfhydryl is destroyed by the time complete inactivation is found. With the technic just described, the effects of ferricyanide, of dilute porphyrindin, and of iodosobenzoate have also been investigated. In urease these reagents in dilute solutions attack only the "a" groups and leave the activity unaltered. In myosin they reacted with the freely reacting –SH groups and brought about 11-16% inhibition of adenosinetriphosphatase activity, in good agreement with the effect of *p*-ClHg benzoate (Table II). Thus, the freely reacting –SH content per cc of myosin was 0.28, 0.28, and 0.27 micromole, by the *p*-ClHg benzoate, ferricyanide, and porphyrindin methods, respectively. The inhibition obtained by this amount of *p*-ClHg benzoate was 11%; porphyrindin (3-fold excess) inhibited only 14%, iodosobenzoate 16%, and ferricyanide (150-fold excess) 11%. (At higher concentrations porphyrindin gave 32% inhibition, Table I.) The gradual inhibition of enzyme activity closely corresponding to the gradual abolition of the –SH groups of myosin is evidence that myosin and adenosinetriphosphatase are identical, as has been postulated by Engelhardt. These results could, however, also be explained on the basis that the enzyme is a small impurity adsorbed on the surface of myosin. It would have to be small enough to escape elec-

trophoretic detection. Furthermore, the properties of the –SH groups of myosin and enzyme would have to be closely similar to explain the experiments reported in Table II and Fig. 1.

The effect of arsenicals on the enzyme was studied as follows. Varying amounts of a 1×10^{-3} *M* solution of mapharsen (3-NH_2, 4-OH phenylarsine oxide)—standardized by KIO_3—were added to 1 cc of 0.97% myosin at pH 7.1, under the conditions outlined above. Five-tenths of a micromole of the arsenical gave no inhibition, and 10 micromoles of arsenoxide produced only 9% inhibition. (Five-tenths of a micromole of *p*-ClHg benzoate inhibited 80%.) Since in most other enzymes we have studied[8] such a marked difference in the effect of this arsenical and of *p*-ClHg benzoate was not found, the possibility arose that the substituents on the benzene nucleus of the compound interfered with the approach of the arsenical to the –SH groups of the enzyme. In agreement with this assumption, 0.5 micromole of the unsubstituted phenylarsine oxide per cc of myosin gave 22% inhibition. At higher concentrations the inhibition by phenylarsine oxide increased.

We are indebted to Dr. Harry Eagle and to Messrs. Parke, Davis and Co. for the generous samples of the arsenicals, and to Dr. L. Hellerman for the *p*-chloromercuribenzoic acid used in this study.

Summary and Conclusions. The inhibition of adenosinetriphosphatase by mercaptide forming compounds and by mild oxidizing agents, and the reactivation by glutathione can be considered as proof that adenosinetriphosphatase is a sulfhydryl enzyme. The close parallelism between the number of –SH groups of myosin attacked by *p*-ClHg benzoate and oxidizing agents and the degree of inhibition of adenosinetriphosphatase activity is evidence in favor of Engelhardt's assumption that myosin and adenosinetriphosphatase are identical.

14618

A New Method of Preventing Blood Coagulation.

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Many anticoagulant substances have been employed for preserving blood in the fluid state. Agents such as oxalates and fluorides act by combining chemically with the available calcium in the blood to form an insoluble calcium compound. Citrates have been employed as the sodium or potassium salt and act by forming calcium citrate, which does not ionize. Heparin has been used to preserve the blood in a fluid state; its action being that of an antithrombin which inhibits the prothrombin necessary for coagulation. Oxalates and fluorides may be employed effectively *in vitro* for maintaining the fluidity of the blood in order that it may be used for certain chemical determinations on either whole blood or plasma. The toxicity of these substances, however, precludes their use in transfusions.

Citrates, particularly sodium citrate, have long been used as anticoagulants in transfusion. Moreover, under present wartime conditions, their employment has increased manifold times, particularly in the preparation of plasma. No untoward effects have attended their clinical use. Heparin, although infrequently employed as anticoagulant in routine transfusions, may prove to be a more ideal agent, but this would be contingent on less expensive methods of manufacture. When blood was collected in paraffin-lined tubes it was found to remain fluid for some time due to the failure of the platelets to disintegrate and release thromboplastin. The same observation was subsequently made when methylmethacrylate tubes were employed.

A method was sought whereby the calcium could be removed by the physical phenomenon of adsorption. In a search for a suitable ion-

adsorbent, a phenol-formaldehyde resin with a polyhydric phenol base was investigated and found to be satisfactory. Several papers[1,2,3] have been published which review in detail the ion-exchange substances. This resin which was commercially available as Amberlite IR-100 had been employed on a large scale in the preparation of ion-free water. The action of this cation-exchange resin in the latter is illustrated as follows:

$$2NaR + CaSO_4 \rightarrow CaR_2 + Na_2SO_4$$
$$2NaR + MgCl_2 \rightarrow 2NaCl + MgR_2$$
$$2NaR + Ca(HCO_3)_2 \rightarrow CaR_2 + 2Na\,HCO_3$$
} Sodium cycle

$$2HR + CaSO_4 \rightarrow CaR_2 + H_2SO_4$$
$$2HR + MgCl_2 \rightarrow MgR + 2HCl$$
$$2HR + Ca(HCO_3)_2 \rightarrow CaR_2 + H_2O + CO_2\ (gas)$$
$$HR + NaCl \rightarrow NaR + HCl$$
} Hydrogen cycle

NaR = Sodium salt of cation-exchange resin.
HR = Hydrogen derivative of cation exchanger.

Preparation of Resin. The cation-exchange resin acts either as a sodium or as a hydrogen exchanger. When employed in the removal of calcium from blood, the resin was placed in the sodium cycle. This was accomplished as follows:

The resin which was available as 50-mesh was placed in a cylindrical separatory funnel with a plug of adsorbent cotton above and below the material. A 5% solution of sodium chloride was then allowed to flow through the material at the rate of 200 cc per cu. inch per minute. Following the adsorption cycle, a back-wash was carried out for 10 minutes. Finally, the resin was rinsed with calcium and magnesium-free water. For the preparation of small portions it was only necessary to stir the resin with successive quantities of 5% sodium chloride solution employing a mechanical stirrer. The supernatant solution was decanted off or removed by filtration. Some of the resin employed was not of an analytical grade and was found to throw color when rinsed with the saline solution. This was eliminated by rinsing the resin with successive portions of boiling water. As successive cycles of regeneration were carried out the color diminished. Sterilization of the resin was accomplished by autoclaving without any deleterious effects.

Theoretically, 1 g of dry resin is sufficient to adsorb 2.2 milliequivalent of calcium. Since the average calcium content of the blood is approximately 5.0 m.e., 2.3 g of resin should have been sufficient to adsorb this quantity of calcium. However, since maximal conditions of adsorption have not thus far been devised, considerably more than this amount of resin was necessary to prevent coagulation of the blood (*cf.* below).

Several methods of collecting the blood were devised. For chemical or serological determinations the blood was mixed directly with the resin in a small bottle, tube, or flask and then obtained free of resin by filtering through an 80-mesh monel-metal or stainless steel screen. Another method adaptable for blood transfusion employed a 250 cc vacuum bottle (Baxter) containing 30 g of resin in the sodium cycle moistened with normal saline. This was autoclaved and the blood was collected in the usual manner. The needle was plunged directly into the vein with the beveled edge away from the vessel wall so that a free flow of blood was obtained. The bottle containing the resin was then inverted so that the blood passed through the resin bed facilitating suitable contact with the resin to assure maximum adsorption of the calcium. Occasional gentle agitation was necessary to avoid partial clotting.

Another method of removing the calcium consisted of filling a glass mantel (6½ x 11/16 inches) threaded on both ends and containing the resin in the sodium cycle. This container held approximately 20 g of the dry resin, which was sufficient to prevent clotting of 150 cc of blood, under the conditions employed. An 80-mesh monel-metal screen was placed at both ends between rubber washers and a glass cannula with a flange was held in place by a metal screw-cap. A 14-gauge needle was attached to one cannula by means of a short length of rubber tubing. A piece of rubber tubing was attached to the other cannula and led into a vacuum bottle through

1 Myers, R. J., Eastes, J. W., and Myers, F. J., *Indust. Eng. Chem.*, 1941, **33**, 697.

2 Myers, R. J., and Eastes, J. W., *Indust. Eng. Chem.*, 1941, **33**, 1203.

3 Harrison, J. W. E., Myers, R. J., and Herr, D. S., *J. Am. Pharm. Assn.*, 1943, **32**, 121.

TABLE I.
Influence of Oxalate or Resin Treatment upon Properties of Blood.

	Specimen	Resin-treated	*Direct or Oxalate
RBC, million/cu mm	1	4.85	4.78
	2	4.76	4.60
	3	3.73	3.52
	4	4.09	4.05
	5	5.21	5.00
Hemoglobin, g/100 cc	1	14.8	14.5
	2	13.5	13.2
	3	12.5	11.6
	4	14.5	14.2
	5	14.0	13.5
Sediment rate, mm/hour	1	3.5	9.2
	2	8.0	13.5
	3	15.0	22.0
Hematocrit, %	1	37.0	32.0
	2	33.0	28.5
WBC, per cu mm	1	6,250	5,200
	2	7,400	6,700
	3	10,500	8,800
Platelet count, per cu mm	1	310,000	260,000
	2	240,000	220,000
	3	375,000	320,000
	4	270,000	250,000
Prothrombin time, sec.	1	16.8	21.2
	2	24.2	25.6
	3	13.6	15.4
	4	15.4	17.2

*Direct refers to finger puncture.

a suction-control valve. The entire apparatus was wrapped and autoclaved. Employing any of the aforementioned methods, the blood was found to remain in a fluid state indefinitely.

The used resin was washed with a 2% sodium carbonate solution, then again washed and regenerated with saline as described previously. This regeneration could be accomplished repeatedly without exhausting the ion-exchange properties of the resin.

Properties of Resin-treated Blood. Blood collected in this manner compared favorably with heparinized blood. It was found satisfactory for hematological, serological, and biochemical examinations. The morphology of the cellular elements of the blood was minimally disturbed. Such tests as the Kolmer-Wassermann, Kahn, Mazzini, complete blood count, hemoglobin, specific gravity, hematocrit, sedimentation rate, total protein, sugar, urea, uric acid, N.P.N., etc., (*cf.* Tables I and II) were successfully performed. Prothrombin times were performed more accurately since no variation in recalcifying the blood was encountered. Blood collected under sterile conditions was satisfactorily employed for culture media in bacteriology.

Four rabbits were bled from the heart and the blood collected in this manner was treated with the resin to prevent coagulation. When the resin-treated blood was then replaced in the rabbits to restore the blood volume no untoward effects were observed.

Summary. 1. A new method of preventing coagulation of blood was devised by the employment of ion-exchange adsorbents. The principle is based on the replacement of the calcium ions in the blood with sodium ions. The most ideal of the ion-exchange agents was found to be a phenol-formaldehyde resin with a polyhydric phenol base. As a calcium-adsorbent the cation-exchange resin was employed in the sodium cycle. 2. Resin-treated blood was employed in hematological, sero-

TABLE II.
Serological and Biochemical Studies of Resin and Oxalate Treated Blood.

Determination	Resin treated*	Oxalated*†
Sugar, mg %	111.2	114.0
Urea nitrogen, %	14.3	13.8
Uric acid, %	4.1	4.4
Total protein, g	7.31	7.25
A/G ratio	2.03	2.16
Albumin, g	4.90	4.75
Globulin, g	2.41	2.20
Fibrinogen	0.268	0.324
Non-protein nitrogen, mg %	29.1	26.4
Inorganic phosphorus, mg %	2.6	2.2
Sugar (24 hours), mg %	22.5	45.0
Specific gravity—whole blood	1.0623	1.0608
" " plasma	1.0285	1.0278
Oxygen capacity, cc	22.73	21.99
Cephalin-cholesterol flocculation test	Negative	Negative
Creatinine, mg %	1.2	1.2
Cholesterol, mg %	182.0	194.0
†Serology:		
Wassermann	Negative	Negative
"	++++	++++
Kahn	Negative	Negative
"	++++	++++
Mazzini	Negative	Negative

* Average of five samples.
† Serum for Serology.

logical and biochemical studies. Minimal alterations in cellular morphology were observed. 3. The resin is inexpensive and can be regenerated innumerable times and reemployed.

Acknowledgment is made to Dr. E. R. Mueller of Resinous Products and Chem. Co., Philadelphia, for the initial supply of the Amberlite IR-100 resin and to Dr. Nessett of Baxter Corporation for preparing the vacuum bottles containing the resin.

14619 P

Vaso-Excitor and -Depressor Substances as "Toxic" Factors in Experimentally Induced Shock*

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In our observations on the visceral circulation of animals in tourniquet and traumatic shock, capillary changes, in addition to those produced by generalized vasoconstriction, were noted remote from the site of injury. This and the fact that similar changes occurred in irreversible hemorrhagic shock led us to look for the presence in the blood of abnormal substances which would account for the observed reactions. Descriptions of the vascular reactions after these shock treatments involving fluid loss are being published.[1] They indicate that the changes are of the order of an initial hyper-reactive condition, suggesting the presence of vaso-excitors, and a

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and New York University. It was also supported in part by the Eli Lilly Research Laboratories and the Josiah Macy, Jr. Foundation.

[1] Zweifach, B. W., Lee, R. E., Hyman, C., and Chambers, R., *Ann. Surg.*, in press.

TABLE I.

Animals	Treatment	Survival after treatment	Time of obtaining blood samples	Reaction of capillary bed in test rat
Dogs	Muscle trauma* (Gregersen's method)	>12 hr	4-6 hr after trauma	vaso-excitor or neutral
		6-8 hr	5-6 hr after trauma (1-1½ hr before death)	vaso-depressor
Rats	Noble Collip drum, 600 turns (no anesthesia)	45 min-2 hr	30-60 min after treatment (45-60 min before death)	" "
Rabbits and Rats	Tourniquet,† duration 3-4 hr	>24 hr	1-6 hr after release	vaso-excitor
"	Tourniquet,† duration 5-7 hr	3-5 hr	30-50 min after release	" "
			1½-4 hr after release	vaso-depressor
Dogs	Graded hemorrhage‡	4-8 hr	Initial reversible period (1-3 hr after bleeding)	vaso-excitor
			Final irreversible period (4-8 hr after bleeding)	vaso-depressor

* Ether administered during period of pounding leg.

† Vascular occlusion of one hind limb. Rabbits were given sodium pentobarbital (20 mg/kg) prior to tourniquet application. Rats were similarly treated just prior to release of tourniquet to permit exteriorization of the mesoappendix.

‡ Bled to produce moderate, followed by drastic, hypotension culminating in a state irreversible to restoration of blood previously withdrawn. Pentobarbital (20-30 mg/kg) or morphine (2-12 mg/kg) administered prior to bleeding.

subsequent hypo-reactive condition in which substances possessing vaso-depressor properties predominate in the blood.

To obtain objective evidence for this, small quantities of blood, taken at intervals during the shock syndrome, were introduced into the blood of normal rats and the effect noted on the vessels of the normal mesoappendix. This test proved to be a discriminating one because of the highly specific pattern of responsiveness of these vessels.

The blood from the shocked animals was either heparinized or allowed to clot and the serum collected and refrigerated for 24 hours. The test rat, under nembutal, was prepared for blood pressure measurements[2] and its mesoappendix exposed for microscopic observation. About 0.5 cc of the blood or serum to be tested was then injected into the tail vein. The effect on the capillary circulation of the test rat was transient but was found to simulate many of the changes previously observed in the shocked animals. For control, it was found that normal and hemolyzed blood or serum of dogs and rabbits after 24 hours refrigeration exerted no specific effects.

The criteria looked for were: (a) altered appearance of the capillary flow with a shift toward either an ischemic or a hyperemic flow and a change in rate of the venular flow; (b) change in responsiveness to topical application of epinephrine; and (c) augmentation or diminution of the vasomotion of arteriolar vessels. The effect on blood pressure was not reliable as a test criterion.

Table I summarizes the types of shock to which the animals were subjected and the reactions of the capillary bed in the test rats to the injections.

The blood of the dogs with muscle trauma was collected by Drs. Gregersen and Root (College of Physicians and Surgeons). The reactions of the blood of dogs which they reported (personal communication) as surviving were either vaso-excitor or neutral. The blood taken 5 to 6 hours after the trauma from dogs which later died gave vaso-depressor reactions.

The fatality of the rats subjected to the Noble-Collip drum was chiefly due to gut injury[3] and the animals were completely comatose when the blood samples were taken.

It is to be noted that the blood samples of the non-fatal cases and the early blood

2 Duncan, G. W., Hyman, C., and Chambers, E. L., *J. Lab. and Clin. Med.*, 1943, **28**, 886.

3 Chambers, R., Zweifach, B. W., and Lowenstein, B. E., *Am. J. Physiol.*, 1943, **139**, 123.

samples of the fatal tourniquet and hemorrhagic cases were all vaso-excitor. This, in general, is the initial reaction to fluid loss.[4] On the other hand, the blood samples taken during the late periods of the fatal shock syndrome were all vaso-depressor.

The effects of known substances were compared with those of the shock depressor blood, noted above. The criteria were diminished responsiveness to epinephrine, loss of vasomotion with the dilator phase persisting and hyperemia.

Normal rats were given intravenous injections of histamine (0.5-3.0 mg/100 g rat), adenylic acid (0.2 mg/100 g), diphosphopyridine nucleotide (3 mg/100 g), Padutin Niphanoid (Winthrop) (5-10 mg/100 g), leukotaxin (1-10 mg/100 g), physostigmine and acetylcholine. All lowered the blood pressure and induced peripheral hyperemia but none gave all the effects of the vaso-depressor serum. For example, Padutin, a kallikrein, slowed the vasomotion, but had no effect on the epinephrine reactivity, while adenylic acid increased the vasomotion.

The vaso-depressor effect of shock plasma or serum was not destroyed by incubation with histaminase (Winthrop T360-N) for 24 hours. That the vaso-depressor was probably not a choline derivative was indicated by the fact that atropinized test rats still gave the vaso-depressor response.

In conclusion, the presence of vaso-excitor substances was demonstrated in the blood of animals during the early period of shock treatment involving fluid loss. Subsequently, when a hypo-reactive state of the capillary circulation occurred, the blood was found to contain vaso-depressor substances having properties which differed from those of a number of known tissue extracts.

[4] Zweifach, B. W., Lowenstein, B. E., and Chambers, R., *Am. J. Physiol.*, in press.

14620

Rancid Fat in Experimental Diets.

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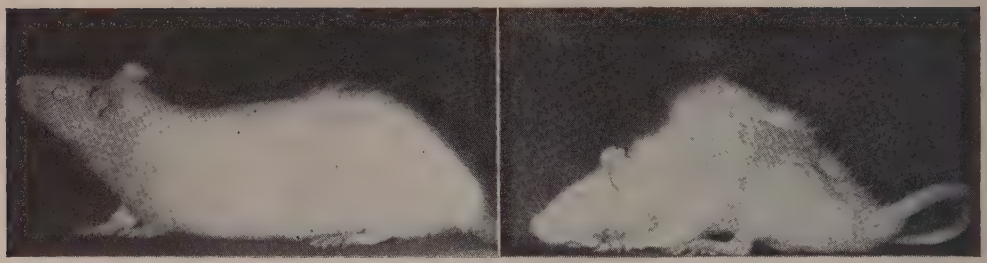
The deleterious effects caused by the ingestion of rancid fats have been reviewed recently by Burr and Barnes.[1] The possible effect of small amounts of rancid fat in the diet on the toxic action of a drug has not been sufficiently emphasized by nutrition investigators. The seriousness of the presence of oxidized fat in the diet was observed in this laboratory when it was found that rats, fed an experimental diet in which corn oil was replaced by lard, developed a humped back and partial paralysis, followed by death. The condition was first noticed in rats fed a diet containing cadmium as a toxic agent; however, it later developed in the control animals of this same series. We observed this abnormal condition in rats fed 6% lard as well as in those fed 25% lard in the diet. In order to interpret our previous data from the cadmium experiment, it was thought advisable to determine what effect, if any, the lard had on the toxicity results.

Experimental. Twenty pairs of albino rats, 21 days of age and equally divided between the sexes, were placed on the following diet: Casein 18%, cornstarch 60%, brewer's yeast 5%, whole dried liver 5%, salt mixture (U.S.P. XII No. 2) 4%, cod liver oil 2%, and lard* or corn oil 6%. Dietary mixtures were prepared in sufficient quantities to last

[1] Burr, G. O., and Barnes, R. H., *Physiol. Rev.*, 1943, **23**, 256.

* The lard used in this experiment was obtained from the Beltsville Experiment Station of the U. S. Department of Agriculture. It was stored at 35°F and was used as needed within a year.

Littermates one year old.



Corn oil in diet. Lard in diet.

FIG. 1.

approximately six weeks and were stored in covered tin buckets in a refrigerator at 35°F. The rats were kept in individual cages and fed and weighed once weekly. The initial weights of the littermates on the lard and corn oil diets respectively varied in no instance more than 2 g.

Results. The marked difference in appearance between the rats in the 2 groups is shown in Fig. 1. The humped back and the roughened coat of the rat on the lard is in sharp contrast to the sleek appearance of the rat on corn oil. The marked emaciation is characteristic of the animals in the lard group. During the rapid growth period of the first 3 months, there was only a slight difference in the growth rates of the 2 groups and no outward symptoms of deficiency in the rats on the lard. Moreover, they did not show the marked symptoms until they had been on the experimental diet for about a year. Before death they developed a partial paralysis, particularly of the hind legs. An animal was able to lift itself with great difficulty up to a food cup, and once with its head in the cup it hesitated to return to a normal position in the cage. The condition was a progressive type of paralysis and had not developed to the extreme stage in all animals at the time of termination of the experiment.

Grossly, the internal viscera of the animals on the diet with lard were small. The uteri were deep brown in color. Microscopically, the significant lesions were uterine pigmentation, tubular atrophy of the testes, and focal degeneration of the voluntary muscles. In the muscles, a moderate number of scattered individual fibers showed varying degrees of vacuolation and gray or tan colored pigmentation (in hematoxylin-eosin stained sections). Occasionally a small number of macrophages were present within a muscle fiber. More pigment was present in macrophages in the interstitial tissues. In the uterus, the pigment was present in muscle cells, in macrophages between the muscle cells, and even in the walls of blood vessels. The pigment had the same appearance as that of the voluntary muscles, and, like the latter, gave no reaction for iron when treated with acid ferrocyanide. The spinal cord and peripheral nerves were not examined.

No anemia was present in the lard group at 14 months. This is in contrast to the effect reported by György *et al.*[2] for a similar diet and confirmed by Burr and Barnes.[1] X-ray photographs revealed no abnormality of the bone structure of the spinal column despite the extreme curvature.

Rancidity of the Lard. The rancidity of the lard, although stored at 35°F, was established by organoleptic tests and the peroxide number was determined. The peroxide number (41.0 millimols per kilo) was high compared to that (2.0 millimols per kilo) of a sample of lard from the same batch but which had been stored at 20°F. By organoleptic test the latter lard was also not rancid. Peroxide numbers were not attempted on the diet stored at 35°F but organoleptic tests on the diet in the food cups were strongly positive for rancidity.

2 György, P., Tomarelli, R., Ostergard, R. P., and Brown, J. B., *J. Exp. Med.*, 1942, **76**, 413.

Discussion. The significant pathological lesions point toward a vitamin E deficiency. Paralysis, with an accompanying emaciation, and failure to increase in weight in adult life are found in vitamin E-deficient rats. The appearance of the voluntary muscles is consistent with that described in the literature[3] as "nutritional myodegeneration" and attributable to vitamin E deficiency. There is some difference in appearance probably because of the slower development of the process in our rats. For example, there is very little coagulation necrosis or inflammatory reaction, and little formation of new muscle fibers, as seen in the less chronic forms. The process appears to have developed earlier in the leg and thigh muscles than in the body-wall muscles. The marked pigmentation of the musculature of the uterus is indicative of prolonged E-depletion.[3]

Although the basic diet is relatively low in vitamin E, it should be noted that the experimental diet included 5% yeast as well as only 6% lard. The early work of Evans and Burr[4] showed that 7.2% lard in the diet did not destroy the vitamin E supplied by wheat germ oil while 22% lard did destroy the vitamin E. Recently, Morris, Larsen, and Lippincott[5] reported that rats fed 50% unheated lard in the diet grew normally. The latter authors made no mention of any antioxidant in their lard. Yeast has been reported[2,6] to have an antioxidant action.

In other experiments we have found that fresh lard is nutritionally equivalent, in our colony of animals, to the vegetable oils which is in agreement with the observations of other laboratories.[7,8]

We wish to emphasize that although a diet may be stored in a refrigerator to prevent oxidation of fat, the storage temperature and length of storage are important. Dietary mixtures should be prepared as frequently as possible and should be stored at a relatively low temperature. We have found that lard did not become rancid within a year when stored at 20°F. Attention is called to the fact that prepared diets fed the animals will become rancid more rapidly than pure fats. Residues in feeding cups should be discarded.

Small amounts of rancid fat in the diet of animals on experiments of long duration may be very important. We found in our earlier experiment with cadmium that rancid fat increased the toxic symptoms. As has been pointed out by Burr and Barnes[1] the deleterious effect of rancid fat is not limited to a deficiency of vitamin E. End products of fat oxidation seem to have toxic actions of their own.

Summary and Conclusions. Studies on the deleterious effects of rancid lard in an experimental diet indicate that it is important to choose fats that will supply the necessary nutrients and not introduce other complicating factors. Rats on diets containing 6% rancid lard developed pathological lesions similar to those found in vitamin E deficiency. These changes were not present in rats receiving a diet in which corn oil replaced the lard. The observations are of particular importance in chronic toxicity studies in which animals are maintained on experimental diets for long periods of time.

3 Mason, K. E., *Yale J. Biol. and Med.*, 1942, **14**, 605.

4 Evans, H. M., and Burr, G. O., *J. A. M. A.*, 1927, **88**, 1462.

5 Morris, H. P., Larsen, C. D., and Lippincott, S. W., *J. Nat. Cancer Inst.*, 1943, **4**, 285.

6 Clausen, D. F., Barnes, R. H., and Burr, G. O., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 176.

7 Evans, H. M., and Lepkovsky, S., *J. Biol. Chem.*, 1932, **96**, 157.

8 Hoagland, R., and Snider, G. G., *U. S. Dept. Agr. Tech. Bull.* No. 821, 1942.

14621

Production of Penicillinase by Bacteria.

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Abraham and Chain[1] first reported the production by *Escherichia coli* and other bacteria of an agent which destroys penicillin. Because of its enzymic nature these workers termed the agent, penicillinase. Hobby *et al.*[2] confirmed the production of this agent by *E. coli*. Harper[3] reported the use of a cellular extract of a paracolon bacillus as a constituent of media for cultivation of bacteria in body fluids of patients treated with penicillin. The penicillin was destroyed, allowing growth of organisms which otherwise would have been inhibited. Lawrence[4] recently reported that the action of takadiastase and clarase in destroying penicillin may be attributed entirely to the presence of contaminating bacteria since penicillinase production is a common property among air contaminants. Studies were carried out in this laboratory to determine the nature and significance of this agent produced by bacteria.[5]

In this study the test used for determining the production of penicillinase by bacteria is similar to that used by Abraham and Chain.[1] It entails the addition of penicillin to growing broth cultures of bacteria or their supernates, and the subsequent testing of the mixture for residual penicillin activity by the Oxford Cup method using *Staphylococcus aureus H* as the test organism. Destruction of the penicillin by a culture serves as an index of penicillinase production by that organism.

Broth cultures of the organisms studied are grown for a minimum of 4 days. To 1.4 cc volumes of these broth cultures is added 0.1 cc of a dilution of penicillin. One cup on each of 3 agar plates previously seeded with *Staph. aureus H* is filled with the penicillin-culture mixture without initial incubation. Five or 6 cultures may be tested on a given plate, although each organism was always run in triplicate. Plates are immediately placed in the 37°C incubator and read at the end of 18 to 24 hours for absence of a zone of inhibition of the *Staph. aureus* indicating destruction of penicillin. The same quantity of penicillin added to a tube of sterile broth and likewise tested serves as a control. The concentration of penicillin is not important as long as the control penicillin broth produces a zone of inhibition on the plate of from 20 to 25 mm in diameter. This concentration must be the same for each culture so studied.

The destruction of penicillin is a rapid process; incubation of the mixture for periods of time ranging from one to six hours before the cups are filled does not materially affect the results. Furthermore, additional incubation actually does occur in the cups.

The results of a typical experiment are shown in Table I. Penicillin-culture mixtures of the penicillinase-positive organisms show either no zone of inhibition or a zone of inhibition considerably less than the control indicating destruction of the penicillin. Sim-

TABLE I.
Detection of Bacterial Penicillinase by an Oxford Plate Technic.

Organism + Penicillin	Zone of inhibition* in mm	Penicillinase
Staph. aureus	24.8	—
Esch. coli	0	+
E. typhosa	25.2	—
Sal. enteriditis	24.9	—
Shig. dysenteriæ	0	+
Shig. paradysenteriæ	14.2	+
Broth control	25.1	

* Inhibition of *Staph. aureus*—average of triplicate tests.

1 Abraham, E. P., and Chain, E., *Nature*, Lond., 1940, **146**, 837.

2 Hobby, G. L., Meyer, K., and Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 277.

3 Harper, G. J., *Lancet*, 1943, **2**, 569.

4 Lawrence, C. A., *Science*, 1944, **99**, 15.

5 Bondi, A., and Dietz, C. C., *J. Bact.*, 1944, **47**, 20.

ilar mixtures of penicillinase-negative organisms show zones of inhibition comparable to that of the control indicating that penicillin was not destroyed by those organisms.

A list of organisms studied by this technic for evidence of penicillinase production is shown in Table II. Among the gram-positive and gram-negative cocci studied no culture was encountered that produced penicillinase. One might have expected these results inasmuch as most infections produced by these organisms respond effectively to penicillin therapy. No doubt penicillinase-producing strains may be found among the cocci, as Abraham and Chain[1] reported finding a Micrococcus which was capable of destroying penicillin. In the group of gram-positive bacilli studied, many of the aerobic spore-formers were positive. All 6 cultures of Clostridia studied were negative.

TABLE II.
Production of Penicillinase by Bacteria.

Organism	Penicillinase
Staph. aureus (5*)	—
Hemo. streptococci (3) (Group A)	—
Viridans streptococci (2)	—
Enterococci (2)	—
Pneumococci (2)	—
N. catarrhalis (2)	—
N. intracellularis	—
N. gonorrheæ	—
B. cereus	+
B. subtilis	—
B. megatherium	+
B. anthracis (2)	+
Clostridia (6)	—
C. diphtheriæ	—
Diphtheroid	—
List. monocytogenes (2)	—
Esch. coli (2)	+
Aerob. aerogenes	+
Paracolon (2)	+
E. typhosa (3)	—
Salmonella (6)	—
Shig. dysenteriæ	+
" *paradysenteriæ* (3)	+
" *paradysenteriæ*	—
" *sonnei* (2)	+
" *newcastle*	+
Proteus vulgaris (3)	—
Alk. fecalis	+
Pseudomonas (3)	—
" (3)	+
Br. bronchiseptica	—
" *melitensis*	—
Hemo. influenzæ (2)	—
" *parapertussis*	—

* Number in parenthesis indicates number of strains tested, otherwise only the single strain was tested.

In the group of gram-negative bacilli, penicillinase-producing cultures were more frequently encountered. All strains of coliform bacilli tested were positive. With the exception of one strain of *Shigella paradysenteriæ* all other members of the genus *Shigella* tested were positive. In contrast, members of the typhoid-salmonella group were uniformly negative. This sharp difference between the two groups might eventually prove useful as a differential aid. It is interesting to note that *Shigella newcastle* whose place in the genus *Shigella* has been questioned by many workers is penicillinase-positive.

Three strains of *Proteus vulgaris* were uniformly penicillinase-negative. Likewise, all non-intestinal, gram-negative bacilli tested, such as Brucella and Hemophilus were also negative.

By use of the same test one may estimate quantitatively the amount of penicillinase produced by different bacteria. The technic is the same except that serial two-fold dilutions of the culture supernates are tested for their ability to destroy penicillin. Because bacteria remaining in the filtrates may continue to produce penicillinase, cultures to be assayed

TABLE III.
Quantitative Estimation of Penicillinase Produced by Different Bacteria.

Organism*	Dilution†	Zone of inhibition in mm
Penicillin control		20.0
Alk. fecalis	1/2	—
	1/4	—
	1/8	13.0
Esch. coli	1/8	—
	1/16	—
	1/32	15.5
B. cereus	1/16	—
	1/32	—
	1/64	15.8
Paracolon	1/32	—
	1/64	—
	1/128	16.2
B. megatherium	1/64	—
	1/128	—
	1/256	14.0

* Ninety-six-hour cultures.
† Two-fold dilutions in 1.5 ml volumes.

must be filtered through bacterial filters or a growth-inhibiting agent must be added. In the experiment shown in Table III, 0.25% phenolized saline was used as the diluting fluid. Phenol in such a concentration did not affect the activity either of penicillin or of penicillinase. These results show that bacteria vary considerably as to the amount of penicillinase they produce.

Discussion. Bacteria capable of producing penicillinase, an enzyme destroying penicillin, are widely distributed in nature. They are common among the gram-negative bacilli normally found in the intestinal tract. With the exception of the Shigella group of organisms most penicillinase-positive organisms are not primary pathogens, a fact of considerable importance.

The relationship of penicillinase production by a bacterium to its resistance to penicillin is a subject of another report.[6] Although there is some correlation between the two phenomena, it is worthy of note at this time that *B. anthracis* which has been reported as susceptible to penicillin is a penicillinase producer.

Otherwise, the significance of penicillinase production by bacteria as it relates to penicillin therapy is open to conjecture. It is conceivable that infected foci secondarily invaded with penicillinase-producing bacteria, such as the coliform bacilli and Pseudomonas, might not respond favorably to penicillin therapy due to destruction of penicillin *in situ*. Further clinical evidence is necessary, however, in order to determine the importance of penicillinase in relation to penicillin therapy. In the meanwhile, it is highly recommended that mixed infections treated with penicillin be tested for the presence of penicillinase-producing bacteria.

As suggested by Rammelkamp and Keefer,[7] penicillinase production in the intestine probably is of considerable importance. It is doubtful whether penicillin could ever be used for treatment of intestinal infections for this reason. Furthermore, it is conceivable that the rapid loss of penicillin following its introduction into patients might be accounted for in part by carriage by the blood stream to the intestinal tract where it is destroyed by bacterial penicillinase.

To date there is no readily available substance that can be used for experimental purposes to destroy penicillin or to inhibit its action. Penicillinase eventually may prove to be this badly needed agent. From a practical point of view a preparation of penicillinase should be as valuable for culturing body fluids containing penicillin as is para-aminobenzoic acid for culturing specimens containing sulfonamides. In this laboratory, culture filtrates containing penicillinase have been used for this purpose with moderate success.

Conclusions. A simple test for the qualitative and quantitative determination of penicillinase produced by bacteria is described. Bacteria capable of producing penicillinase are widely distributed in nature; such organisms vary considerably as to the amount of penicillinase they produce. The possible significance of penicillinase and penicillinase-producing bacteria in relation to penicillin therapy is discussed.

[6] Bondi, A., and Dietz, C. C., PROC. SOC. EXP. BIOL. AND MED., 1944, submitted for publication.

[7] Rammelkamp, C. H., and Keefer, C. S., *J. Clin. Invest.*, 1943, **22**, 425.

14622

Relationship of Penicillinase to the Action of Penicillin.

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In a previous report[1] a survey was made of the common pathogenic and non-pathogenic organisms to determine their ability to produce penicillinase, an enzyme which destroys penicillin. Bacteria capable of producing this enzyme were found to be rather common, particularly among the Bacillus, Shigella, and coliform groups of bacteria. Most of the organisms commonly recognized as primary pathogens failed to produce penicillinase, regardless of their sensitivity or insensitivity to penicillin. Furthermore, two penicillinase-positive organisms were encountered that were slightly susceptible to penicillin. Similar observations led Abraham and Chain[2] to conclude that production of penicillinase by bacteria probably was not the sole factor in determining their resistance or sensitivity to penicillin. As a result of these observations further investigation as to the significance of penicillinase as a factor in the mode of action of penicillin appeared warranted.

A number of organisms previously tested for penicillinase production[1] were studied to determine their relative susceptibility to penicillin. To tubes of extract or infusion broth were added dilutions of penicillin to give final concentrations of 5.0, 0.5, and 0.05 units per cc. A set of broth tubes containing these 3 concentrations of penicillin was inoculated with a drop of a 1/100 dilution of a 24-hour broth culture of the organism being studied. The results were read at the end of 24 hours for inhibition of growth.

The relationship between the susceptibility of an organism to penicillin and the ability of the organism to produce penicillinase based on these experiments is shown in Table I. Only a few of the organisms falling into each of 5 distinct categories are listed. Of particular note is the fact that not all penicillinase-negative organisms are susceptible to penicillin. Although organisms such as staphylococci, hemolytic streptococci, and gonococci were susceptible to low concentrations of penicillin, concentrations ten to a hundredfold greater were required to inhibit members of the typhoid-salmonella group. Furthermore, even the highest concentrations used would not inhibit certain other penicillinase-negative organisms such as Pseudomonas and *Brucella melitensis*. It may be concluded, therefore, that the inability of an organism to produce penicillinase is not necessarily a determining factor in its sensitivity to penicillin.

Among the penicillinase-positive organisms, 21 of 23 strains tested were not inhibited by penicillin in a concentration of 5.0 units per cc. The remaining two strains were inhibited by this concentration in spite of their ability to produce penicillinase. No organism was found among the penicillinase-positive cultures that was inhibited by concentrations less than 5.0 units per cc. It appears as though penicillinase production by a bacterium is an important factor in its resistance to penicillin, although not necessarily the only factor.

A number of workers[3,4,5] have succeeded in developing penicillin-resistant strains of bacteria by cultivation in increasing concentrations of penicillin over long periods of time. As yet the nature of this acquired resistance is not known. A possible factor in the development of this resistance could be the acquisition by the organism of the ability to produce

1 Bondi, A., and Dietz, C. C., PROC. SOC. EXP. BIOL. AND MED., 1944, submitted for publication.

2 Abraham, E. P., and Chain, E., *Nature*, Lond., 1940, **146**, 837.

3 McKee, C. M., and Rake, G., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 275.

4 Rammelkamp, C. H., and Maxon, T., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 386.

5 Spink, W. W., Ferris, V., and Vivino, J. J., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 207.

TABLE I.
Relationship of Susceptibility to Penicillin with Destruction of Penicillin by Bacteria.

Organisms*	No. of cultures encountered	Penicillinase	Inhibition of growth Units/ml of penicillin 0.05	0.5	5.0
Staph. aureus Hemo. strept. *N. gonorrheæ*	20	—	+ —	+	+
E. typhosa Salmonella *Prot. vulgaris*	14	—	—	—	+
Pseudomonas *Br. melitensis*	8	—	—	—	—
E. coli *B. cereus* *Shig. dysenteriæ*	21	+	—	—	—
Alk. fecalis *B. anthracis*	2	+	—	—	+

* Organisms listed do not include all the species encountered in each category.

TABLE II.
Failure of Organisms Made Resistant to Penicillin *in Vitro* to Produce Penicillinase.

Organism	No. of transplants in penicillin	Units/ml of penicillin inhibiting growth	Penicillinase production
Staph. aureus (X-3)	0	0.045	—
	6	0.09	—
	10	0.19	—
	17	0.75	—
	22	3.0	—
	27	12.0	—
E. typhosa (Panama)	0	1.5	—
	6	6.0	—
	12	24.0	—
	23	48.0	—
P. vulgaris (T 3028)	0	3.0	—
	4	6.0	—
	10	12.0	—
	18	48.0	—

penicillinase, although evidence to the contrary has been reported.[4]

In order to confirm the latter report, strains of *Staph. aureus, Eberthella typhosa,* and *Proteus vulgaris* were rendered resistant *in vitro* by cultivation in increasing concentrations of penicillin. Transplants were made at 2- to 3-day intervals in tubes of broth containing increasing amounts of penicillin over a period of 6 to 8 weeks.

Isolations made at different stages of the development of resistance were stored under mineral oil and tested at the same time for susceptibility to penicillin. All such isolations were tested for their ability to produce penicillinase as shown in Table II. In spite of the acquisition of resistance, none of the cultures produced penicillinase.

Discussion. The development of sulfonamide-fast bacteria has been reported by many workers.[6,7,8,9] In the case of *Staph. aureus* this resistance has been associated with an

[6] MacLeod, C. M., and Doddi, G., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 69.

[7] Westphal, L., Charles, R. L., and Carpenter, C. M., *Ven. Dis. Inform.*, 1940, **21**, 183.

[8] Strauss, E., Dingle, J. H., and Finland, M., *J. Immunol.*, 1941, **42**, 331.

[9] Vivino, J. J., and Spink, W. W., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 336.

increased synthesis of para-aminobenzoic acid.[10] That the same mechanism is not involved in the development of penicillin-fastness is evidenced by susceptibility of sulfonamide-fast strains of staphylococci and pneumococci[5,11] to penicillin. Furthermore, it is evident that penicillinase does not hold analogous relationship to penicillin-fast staphylococci as para-aminobenzoic acid does to sulfonamide-fast staphylococci. Other factors as yet unknown must be concerned in the development of resistance by bacteria to penicillin.

That development of resistance to penicillin is not dependent on penicillinase is not surprising inasmuch as inability to produce penicillinase does not appear to be the determining factor in the sensitivity of an organism to penicillin. The factors concerned in the development of resistance to penicillin by bacteria no doubt are related to the factors which determine their susceptibility to penicillin.

It is to be expected that organisms producing penicillinase would not be highly susceptible to penicillin. The susceptibility of the two penicillinase-positive organisms to higher concentrations of this antibiotic may be attributed to production of penicillinase in smaller amounts. Furthermore, 24-hour cultures were used in the susceptibility tests whereas cultures 4 days or older contain much greater amounts of penicillinase.

Conclusions. Inability to produce penicillinase is not a determining factor in the sensitivity of an organism to penicillin. However, penicillinase-producing bacteria are not likely to be highly susceptible to penicillin. Development of resistance to penicillin by a bacterium is not associated with acquisition by the bacterium of the ability to produce penicillinase.

[10] Dandy, M., Larkum, N. W., Oswald, E., and Streightoff, F., *Science*, 1943, **97**, 265.

[11] Powell, H. M., and Jamieson, W. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 387.

14623

Production of Labyrinthine Paralysis by Application of Local Anesthetics to External and Middle Ear.*

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In the course of experiments dealing with other problems of abnormal labyrinthine excitability, methods of eliminating the impulses from the labyrinth were developed that may be able to make more radical measures (Dandy,[1] Putnam,[2] Mollison[3]), at least in some instances of Ménière syndrome, unnecessary. While Brown-Séquard's[4] procedure of producing symptoms of labyrinthine paralysis by instillation of chloroform into the external auditory canal in guinea pigs is not applicable to cats or dogs, paralysis of the labyrinth was obtained, in cats, by electrosmosis of solutions of local anesthetics introduced into the external auditory meatus. Two per cent solutions of cocain, pontocain, or metycain in 80% alcohol were instilled into the external meatus, and the meatus was plugged by an electrode that was wrapped by cotton soaked with the

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Temple University.

[1] Dandy, W. E., *Acta Otolaryngol.*, 1934, **20**, 1.

[2] Putnam, T. J., *Arch. Otolaryngol.*, 1938, **27**, 161.

[3] Mollison, W. W., *J. Laryng. and Otol.*, 1936, **51**, 38.

[4] Brown-Séquard, C. E., *C. R. Soc. de biol.*, Ser. 7, 1880, **2**, 383.

same solution. The ear electrode was connected to the anode, an indifferent electrode fastened to the abdomen was connected to the cathode of a constant current. When 5 milliamperes were applied for 5-10 minutes, typical symptoms of labyrinthine paralysis appeared such as nystagmus to the opposite side, rotation of the head (see Fig. 1), pleurostonus, circus movements, falling to the same side. These symptoms persisted for from 3-4 hours. When the cocain was bilaterally introduced into the labyrinths by this method, a transient definite reduction of the labyrinthine excitability to rotatory stimuli could be obtained (*e.g.*, duration of the postrotatory nystagmus in cat 39, 13-17 sec. before, 6-9 sec. 20 min. after bilateral cocain electrosmosis; in cat 40, 12-15 sec. before, 3-6 sec. 10 min. after this procedure; next day normal values). In dogs and in man, it was not possible to obtain symptoms of labyrinth paralysis by electrosmosis of cocain solutions.

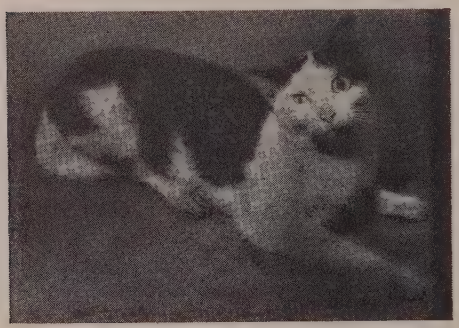

FIG. 1.
Cocaine-electro-endosmosis of right ear 3:33-3:43. Photographed at 4:36. Note, besides abnormal position of head, rightsided Horner (paralysis of sympathetic fibers to eyeball on their way over promontory).

† R. Magnus and A. de Kleyn (Bethe's Handb. d. Physiol., 1926, **11**, 875) erroneously quote C. J. König (Contribution a l'étude expérimentale des canaux semicirculaires. Thèse de Paris, 1897) and J. Breuer (Sitzungsber. d. Akad. d. Wissench. Wien, Mathem. naturw. Kl. III, 1905, **112**, 315) as observing labyrinthine paralysis following injection of cocain into the middle ear in guinea pigs. These two authors applied cocain to the semicircular canals directly, in pigeons. For "cats, etc." Magnus and de Kleyn recommended only injection of cocain into the labyrinth.

Therefore, it was determined whether substances injected through the drum into the cavum tympani are able to permeate into and to influence the activity of the labyrinth.† The experiments were performed on dogs and cats, and the following substances were tested: alcohol 95% 1-2 cc, alcohol-glycerin ãa 0.5-1 cc, alcohol-physiologic NaCl ãa 0.5, alcohol 0.5 cc plus vasoconstrictors (0.5 cc ¼% neosynephrin, 1% propadrine HCl, or 1% paredrine HBr), 0.5 cc 2% cocain solution in 80% alcohol with equal amounts of the above-mentioned vasoconstrictors. All these substances were able to produce symptoms of labyrinth paralysis, except injections of the vasoconstrictors alone (diluted by equal amounts of saline).

The paralytic effect as indicated by nystagmus to the normal side appeared within 10-45 minutes; its duration varied. It was shortest (about 1 hour) after injection of 95% alcohol diluted with equal parts of physiologic NaCl solution, longest after injection of alcohol plus the vasoconstrictors or 2% alcoholic cocain solution plus equal amounts of vasoconstrictors (above 5 hours). All these effects were transient, the animals showing no manifest symptoms of labyrinth paralysis on the day following the injection. Repeated intratympanic injections, *e.g.*, of alcohol on 5 successive days, produced each time a transient nystagmus of several hours' duration.‡ However, even after a single intratympanic injection of alcohol or of an alcoholic cocain solution the tonic impulses from the affected labyrinth seem to be somewhat diminished for a week. This is indicated by the fact that an injection of the same solution into the opposite ear after an interval of several days produces a nystagmus of shorter duration and lower frequency than the first injection (*e.g.*, after injection of 2% cocain in 80% alcohol, 1% propadrine HCl ãa 0.5 into the left ear: nystagmus to right, duration 5

‡ Occasionally the injections failed to produce the labyrinth paralysis or repeated injections were necessary, probably because the fluid escaped through the Eustachian tube. This factor may also interfere in man and may necessitate plugging of the pharyngeal ostium of the tube. These problems of clinical application are now being studied with Dr. M. Ersner.

hours, frequency 140 decreasing to 108; after injection into right ear several days later: nystagmus to left 3½ hours' duration, frequency 102 decreasing to 76).

Summary. In cats, paralysis of the labyrinth can be obtained by electrophoresis of cocain solutions from the external auditory meatus. In cats and dogs, such an effect can be produced by injection of local anesthetics into the tympanic cavity. The effect can be prolonged by combination of the local anesthetic with vasoconstrictors. The use of such intratympanic injections for the treatment of abnormal states of labyrinthine irritation is proposed.

14624 P

Insensible Perspiration and Keratinization Process.

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The keratinization of the epidermis is associated with considerable dehydration since the Malpighian layer contains 70% to 80% water, whereas the water content of the horny layer does not exceed 30%.[1] If the liberated water is lost with the insensible perspiration, conditions in which keratinization is accelerated must show increased insensible perspiration values. Earlier observations seemed to be in accord with this supposition.[2,3] Lately, Pinsen[4] found 2- to 3-fold values of insensible perspiration in dry, scaling skin. Further experiments on this subject are reported in the present study.

Method. The insensible perspiration was measured by absorbing water vapor from the skin in linen bags filled with anhydrous calcium chloride. These bags were weighed, suspended under glass bells which were firmly taped to the skin, and weighed again after 60 minutes.*

Results. Parallel values obtained in simul-

TABLE I.
Insensible Perspiration in Psoriasis.
Measurement on Adjacent Areas of Normal Skin and Psoriasis Patches.
Diameter of glass bells 4.71 cm.

Date	Name	Sex	Age	Region	Room temp., F°	I.P. in mg		Increase of I.P. in psoriasis
						Normal skin	Psoriasis skin	
11-27-43	H.H.	M	42	Back	—	9.4	73.4	8x
12-28	L.	M	43	Abdomen	75	23.8	109.1	4x
2- 7-44	C.E.	M	77	Chest	77	—	92.3	—
2- 8	E.M.	M	56	”	76	13.1	88.9	6x
2-12	E.M.	M	56	Abdomen	74	—	58.4	—
2-19	D.S.	M	66	Sacrum	76	25.5	98.6	4x
2-22	—	M	—	Back	—	18.6	55.5	3x
3-11	A.K.*	M	33	Abdomen	77	—	97.4	—
3-11	A.K.*	M	33	”	77	—	90.8	—
4-17	M.R.†	F	40	Thighs	77	14.7	56.3	4x

* Two adjacent areas measured in generalized psoriasis.
† Symmetrical areas measured.

1 Rothman, S., and Schaaf, F., Chemie der Haut, *Jadassohn's Hand. d. Haut- u. Geschlechtskr.*, 1929, **1**, 161, J. Springer, Berlin.

2 Rothman, S., *Strahlentherapie*, 1930, **35**, 381.

3 Rothman, S., *Zentralbl. f. Haut- u. Geschlechtskr.*, 1931, **37**, 30.

4 Pinsen, E. A., *Am. J. Physiol.*, 1942, **137**, 492.

* A detailed description of the method is given in Felsher, Z., Hereditary Ectodermal Dysplasia, *Arch. of Derm. and Syphil.*, in press.

TABLE II.
Insensible Perspiration in Exfoliative Dermatitis.
36-year-old white male suffering from generalized exfoliative dermatitis following arsphenamine medication. All measurements were made on abdomen.

Date	I.P. values in mg Right	Left	Remarks
12- 6-43	216.5	—	Abundant lamellous scaling.
12- 7	222.5	229.2	Same.
12-24	60.5	68.7	Scaling greatly diminished.
1-20-44	21.3	19.7	Skin completely cleared up. No scaling.

TABLE III.
Insensible Perspiration in Ichthyosis Vulgaris.

Date	Name	Sex	Age	Region	I.P. in mg	Room temp., F°	Remarks
12-19-43	A.T.	F	51	Rt. thigh Rt. poplit. fossa	23.3 24.8	77	Popliteal area free of ichthyosis
1-20-44	A.L.	F	34	Left thigh Left leg	29.6 27.0	—	Adjacent ichthyotic areas
2- 7	M.S.	F	13	Rt. thigh Left thigh	34.4 34.2	77	Symmetrical ichthyotic areas

TABLE IV.
Insensible Perspiration in Ultraviolet Erythema.

Date	Name	Sex	Age	Region	I.P. in mg Normal skin	Eryth. skin	Room temp., F°	Remarks
3- 1-44	M.M.	F	19	Abdomen	13.8	17.9	—	Mild erythema. Increase of I.P. in erythematous area 30%. Symmetrical areas used.
3- 7	M.M.	F	19	"	20.9	28.9	—	Vivid erythema. Increase 38%. Symmetrical areas.
3- 8	S.R.	M	50	Thighs	24.2	33.8	78	Mild erythema. Increase 40%. Symmetrical areas.
4-21	S.R.	M	50	Abdomen	12.8	16.4	75	Increase 20%. Temp. of erythematous area 0.8°C higher than that of normal skin.

taneous measurements of symmetrical or adjacent normal skin areas in the same person were equal within 10% in five experiments. Measurements over *psoriasis* patches with hyperproduction of keratin showed an increase of 3 to 8 times the values of normal adjacent areas (Table I). In *exfoliative dermatitis* with greatly accelerated keratinization a 10-fold increase was obtained at the height of the eruption, and gradual decrease to normal values on recovery (Table II). In *ichthyosis vulgaris* the thickening of the horny layer is not associated with increased proliferation of the Malpighian layer, and the hyperkeratosis seems to be due merely to a disturbance in the separation of the scales. Table III shows practically no or very slight increase of insensible perspiration in this disease.

In psoriasis and exfoliative dermatitis increased keratinization is associated with arterial hyperemia. However, experiments on ultra-violet erythema reveal that arterial hyperemia in itself, without increase of keratin formation, causes only a slight increase in

TABLE V.
Insensible Perspiration in Post-inflammatory Scaling.

Date	Name	Age	Sex	Region	I.P. in mg: Normal area	I.P. in mg: Scaling area	Room temp., F°	Increase in scaling area	Remarks
3-10-44	M.M.	19	F	Abdomen	19.5	44.8	77	2.3x	Measured 4 days after U.V.L. irradiation. No erythema. Moderate fine scaling.
4-31	S.R.	50	M	"	19.0	42.1	71	2.2x	Measured 9 days after U.V.L. irradiation. Lamellous scaling. Skin temp. of scaling area same as that of normal area.

insensible perspiration (Table IV). A marked increase is noted after the onset of scaling when the inflammation has subsided (Table V).

Conclusions. Hyperproduction of keratin in the epidermis causes an increase of insensible perspiration. It seems probable that physiologically, too, one part of the insensible perspiration is contributed by the keratinization process in consequence to the dehydration of Malpighian cells.

14625

Germicidal Potency and Tissue Toxicity of Surface Active Saline Mixture of Azochloramid.

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From the Department of Bacteriology, University of California, Los Angeles.

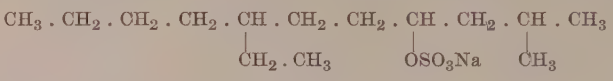
In previous communications[1,2] results were reported on the germicidal efficiency of the organic chlorine compound Azochloramid. The germicide was tested for its effect on living embryonic chick heart tissue fragments cultivated *in vitro* as well as for its ability to kill the test organisms *Staphylococcus aureus* and *Eberthella typhosa*. The tissue fragments and bacteria were exposed separately to the germicidal dilutions for 10 minutes at 37°C in the presence of a standard amount of organic matter. The organic matter was composed of a mixture of 3 parts embryonic extract and 1 part horse serum. A number known as the toxicity index was calculated which was defined as the highest dilution of germicide required to kill the tissue cells in 10 minutes to the highest dilution required to kill the test organisms in the same period of time under identical conditions. Theoretically, an index less than 1 means that the germicide is more toxic to the bacteria than to the embryonic chick heart tissue fragments; an index greater than 1 means that the germicide is more toxic to the tissue cells than to the bacteria.

Recently Azochloramid has been improved by the addition of sodium tetradecyl sulfate, a negatively charged (anionic) surface active or wetting agent. Chemically it is the sodium

[1] Salle, A. J., McOmie, W. A., Shechmeister, I. L., and Foord, D. C., *J. Bact.*, 1939, **37**, 639.

[2] Salle, A. J., Shechmeister, I. L., and McOmie, W. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 614.

salt of the sulfuric acid ester of the aliphatic alcohol, 2-methyl-7-ethylundecanol-4. Its structural formula is as follows:



Sodium 7-ethyl-2-methyl-undecyl-4 sulfate

Sodium tetradecyl sulfate is a transparent, colorless and odorless, waxy substance. A saturated aqueous solution of the compound has a surface tension of 30 dynes/cm as compared to 72 dynes/cm for pure water. The fact that sulfates are derivatives of much stronger acids than soaps explains why sodium tetradecyl sulfate is neutral and remains active in acidic and strong salt solutions where soaps cannot be used.

The combination of sodium tetradecyl sulfate with Azochloramid produces a solution that gives greater penetration into recesses and crypts of infected tissue and clumps of bacteria. The wetting agent is effective in establishing close contact between Azochloramid and bacteria.

It was considered of great interest and importance to determine what effect the addition of the wetting agent would have on the germicidal potency and tissue toxicity of Azochloramid. The same method was employed as previously reported.[3]

Effect of Wetting Agent on Tissue. The wetting agent produced no inhibitory effect on the growth of embryonic chick heart tissue fragments when used in dilutions of from 1:500 to 1:2500 in a period of 10 minutes at 37°C and in the presence of a standard amount of organic matter. The most convenient and generally useful preparation recommended by the manufacturer* is one that contains Azochloramid in a concentration of 1:3300 and sodium tetradecyl sulfate in a concentration of 1:1000.

It may be concluded that the concentration of wetting agent present in such a preparation is insufficient to produce any tissue toxicity when applied clinically.

Effect of Azochloramid on Tissue and Bacteria. The highest dilution of Azochloramid with wetting agent that killed embryonic chick heart tissue at 37°C in the presence of a standard amount of organic matter was found to be 1:18,000. The highest dilution of Azochloramid with wetting agent that killed *Staphylococcus aureus* and *Eberthella typhosa* under the same conditions was 1:70,000 and 1:108,000 respectively. The results give toxicity indexes of 0.26 for *Staphylococcus aureus* and 0.17 for *Eberthella typhosa.* The toxicity indexes previously reported for Azochloramid without wetting agent were 0 3 for *Staphylococcus aureus* and 0.08 for *Eberthella typhosa.* The results are recorded in Table I.

Conclusions. The addition of sodium tetradecyl sulfate to Azochloramid increased the efficiency of the germicide for *Staphylococcus aureus* and decreased it for *Eberthella typhosa.* Since *Staphylococcus aureus* is likely to be the better test organism it may be concluded that the addition of wetter probably improved the germicidal efficiency of Azochloramid. Certainly the addition of wetter greatly improved Azochloramid by decreasing the concentration

TABLE I.
Effect of Sodium Tetradecyl Sulfate on Germicidal Efficiency of Azochloramid.

Highest killing dilution and toxicity index of Azochloramid without wetting agent				Highest killing dilution and toxicity index of Azochloramid with wetting agent			
Tissue (A)	*Staphylococcus aureus* (B)	*Eberthella typhosa* (B)	Toxicity index (A/B)	Tissue (A)	*Staphylococcus aureus* (B)	*Eberthella typhosa* (B)	Toxicity index (A/B)
1:7800	1:26,000		0.3	1:18,000	1:70,000		0.26
1:7800		1:101,000	0.08	1:18,000		1:108,000	0.17

[3] See papers referred to above for method of performing the test.

* Wallace and Tiernan Products, Inc., Belleville, N.J.

TABLE II.
Effect of Germicides on Embryonic Chick Heart Tissue and Bacteria.
Highest killing dilution for tissue and bacteria and corresponding toxicity index.

Staphylococcus aureus				*Eberthella typhosa*			
Germicide	Tissue (A)	Bacteria (B)	Toxicity index (A/B)	Germicide	Tissue (A)	Bacteria (B)	Toxicity index (A/B)
Halogens:				Halogens:			
Iodine	1:650	1:3,520	0.2	Azochloramid	1:18,000	1:108,000	0.17
Azochloramid	1:18,000	1:70,000	0.26	Iodine	1:650	1:3,370	0.20
Chloramine	1:900	1:2,000	0.45	Chloramine	1:900	1:2,000	0.45
Phenols:				Silver compounds:			
Hexylresorcinol	1:1,450	1:1,620	0.9	Silver lactate	1:100	1:690	0.15
P-hydroxyphenyl-n-amyl sulfide	1:2,800	1:2,700	1.0	" citrate	1:610	1:4,500	0.14
O-n-hexylphenol	1:3,125	1:2,900	1.1	" nitrate	1:140	1:1,250	0.11
P-hydroxy-diphenyl sulfide	1:3,125	1:2,500	1.3	" protein strong	1:25	1:175	0.14
Amphyl	1:350	1:300	1.15	Silver protein mild	1:30	1:70	0.43
Phenol	1:224	1:110	2.0	Silver picrate	1:250	1:350	0.71
Silver compounds:				Phenols:			
Silver lactate	1:100	1:110	0.9	Hexylresorcinol	1:1,450	1:1,770	0.8
" citrate	1:610	1:600	1.0	P-hydroxyphenyl-n-amyl sulfide	1:2,800	1:2,700	1.0
" picrate	1:250	1:225	1.1	O-n-hexylphenol	1:3,125	1:2,800	1.1
" nitrate	1:140	1:80	1.8	P-hydroxy-diphenyl sulfide	1:3,125	1:3,125	1.0
" protein strong	1:25	1:15	1.7	Amphyl	1:350	1:300	1.15
Silver protein mild	1:30	1:12*	2.5+	Phenol	1:224	1:186	1.2
Mercurials:				Mercurials:			
Metaphen	1:2,070	1:1,370	1.5	Metaphen	1:2,070	1:5,660	0.4
Mercurochrome	1:90	1:12½*	7.2+	Mercurochrome	1:90	1:140	0.6
Merthiolate	1:4,220	1:25*	169.0+	Merthiolate	1:4,220	1:2,660	1.6
Dyes:				Dyes:			
Brilliant green	1:33,600	1:5,500	6.0	Brilliant green	1:33,600	1:208	161.0
Crystal violet	1:60,800	1:2,800	21.8	Crystal violet	1:60,800	*	—
Methyl " B	1:25,000	1:1,200	20.8	Methyl " B	1:25,000	*	—
" " 6B	1:25,000	1:1,150	21.4	" " 6B	1:25,000	*	—
Methylene blue	1:7,000	*	—	Methylene blue	1:7,000	*	—
Proflavine	1:3,200	*	—	Proflavine	1:3,200	*	—
Acriflavine base	1:3,000	*	—	Acriflavine base	1:3,000	*	—
" HCl	1:2,700	*	—	" HCl	1:2,700	*	+

* Failed to kill.

required to produce the same effect as germicide without wetter. This results in a considerably more potent and economical preparation.

For purposes of comparison all germicides tested to date are included in Table II. The halogens gave the lowest toxicity indexes when tested against both *Staphylococcus aureus* (Gram positive) and *Eberthella typhosa* (Gram negative). Since *Staphylococcus aureus* is likely to be the better test organism the halogens were followed by the phenols, the silver compounds, the mercurials, and lastly the dyes in the order named.

14626 P

The Action of Xanthopterin on Tumor Growth.

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In a recent paper[1] we reported that a "folic acid concentrate" and the crystalline *L. casei* factor which seems to be related to folic acid were found to be strong inhibitors of tumor growth. As a possible relationship of folic acid to Xanthopterin (the pigment of yellow butterfly wings) has been suggested by a number of investigators,[2,3,4,5] it seemed of interest to test the action of synthetic Xanthopterin on tumor growth. Xanthopterin was assayed as described previously by Laszlo and Leuchtenberger.[6] In Table I an experiment is presented in which varying doses of Xanthopterin were studied. It is evident from this table that Xanthopterin is a strong inhibitor of tumor growth. The degree of inhibition depends on the dose injected, and its potency is comparable to that of the *L. casei* factor. Similar results were obtained in 30 experiments in which 214 animals were used.

Leukopterin* (the pigment of the white butterfly) was tested by the same method. It did not cause inhibition of tumor growth even in a dose 100 times larger than that of Xanthopterin. In this connection it might be of interest to quote the observations of Wright and Welch.[5] They reported activity of Xanthopterin and inactivity of Leukopterin in the synthesis of folic acid by rat liver *in vitro*.

In the course of investigations concerned with substances antagonistic to tumor growth inhibitors, as suggested by J. C. Keresztesy, Leukopterin was tested. In Table II one experiment is presented in which the effect of Xanthopterin is compared with that of a combination of Xanthopterin and Leukopterin.

It may be seen from this table that Leukopterin neutralizes the action of Xanthopterin.

In view of the tumor growth inhibitory properties of the *L. casei* factor and of Xanthopterin, experiments are in progress in which mice bearing spontaneous breast adenocarcinomas are treated with these substances. Though no definite conclusions can be drawn so far the changes in these tumors are most striking. After a few intravenous injections some hard tumors are changed into soft bags

TABLE I.
Effect on Tumor Growth of 4 Intravenous Injections of Xanthopterin in Varying Doses Given Over a Period of 48 Hours at 12 Hours Interval.* Seven animals were used in each group.

Group No.	Dose of Xanthopterin, γ	Mean terminal tumor wt, mg	Standard error, mg	Significance ratio
980	0	866	47	—
979	0.031	674	33	3.4
978	0.125	606	27	4.8
977	0.500	513	38	5.9
976	2.000	389	23	9.1

* Female Rockland mice transplanted with Sarcoma 180; start of the experiment 8 days after transplantation; mice kept on normal diet.

1 Leuchtenberger, C., Lewisohn, R., Laszlo, D., and Leuchtenberger, R., Proc. Soc. Exp. Biol. and Med., 1944, **55**, 204.

2 Mitchell, H. K., *Science*, 1943, **97**, 442.

3 Totter, J. R., and Day, P. L., *J. Biol. Chem.*, 1943, **147**, 257.

4 Totter, J. R., Shukers, C. F., Kolson, J., Rims, V., and Day, P. L., *J. Biol. Chem.*, 1944, **152**, 147.

5 Wright, L. D., and Welch, A. D., *Science*, 1943, **98**, 179.

6 Laszlo, D., and Leuchtenberger, C., *Cancer Research*, 1943, **3**, 401.

* We received the synthetic Leukopterin through the courtesy of Drs. E. L. R. Stokstad, B. L. Hutchings, J. H. Boothe, and J. H. Mowat of Lederle Laboratories, Inc..

TABLE II.

Effect on Tumor Growth of 4 Intravenous Injections of Xanthopterin and of a Mixture of Xanthopterin and Leukopterin Given Over a Period of 48 Hours at 12 Hours Interval.* Seven animals were used in each group.

Group No.	Material injected	Dose, γ	Mean terminal tumor wt, mg	Standard error, mg	Significance ratio
959	Saline	—	577	42	—
956	Xanthopterin	0.5	341	21	to 959: 5.0 to 957: 5.3
957	Xanthopterin + Leukopterin	0.5 1.0	620	48	

* Female Rockland mice transplanted with Sarcoma 180; start of the experiment 10 days after transplantation; mice kept on normal diet.

containing necrotic tissue. Detailed descriptions, both macroscopic and microscopic, will be presented in a subsequent paper. Daily injections of 0.5 γ or 5γ respectively of *L. casei* factor or of 10 γ Xanthopterin were given. The animals were kept on a normal diet.

Summary. Xanthopterin inhibits tumor growth. Leukopterin does not inhibit tumor growth. It neutralizes the effect of Xanthopterin.

We gratefully acknowledge the cooperation of Dr. J. C. Keresztesy and Dr. J. E. Little of the Research Laboratories of Merck & Co., Inc.

14627 P

Unidentified Inclusions Within the Erythrocytes in Certain Cases of Febrile Anemia.

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This brief report concerns 3 cases of progressive febrile anemia, 2 ending fatally, which were characterized by the presence within the red cells of unidentified coccoid and bacillary bodies. These were stained dark blue or bluish purple with Wright or Giemsa stains (Fig. 1). From one to 20 or more bodies were present in a single cell, and from 10 to 55% of the cells were affected. They were somewhat pleomorphic, but frequently appeared in the form of diploids, tetrads, or short batonnets, straight or slightly curved. The presence of the structures was often associated with a de-hemoglobinization of the cells; in such cases, they were clustered at the margin.

In unstained preparations, the bodies were colorless, very slightly refractile and possessed of an oscillatory motion within the cell. Treated with HCl and potassium ferrocyanide, they gave a positive iron reaction; the iron staining persisted in acid solutions, but disappeared after exposure to alkali at about pH 10. The morphology of the bodies was not altered by these procedures. They were not blackened by $(NH_4)_2S$, as is hemosiderin, nor by silver nitrate; they gave no histochemical reaction for alkaline phosphatase; the Feulgen reaction was also negative. They were resistant to autolysis, and to tryptic digestion.

By laking the blood with distilled water or saponin, it was possible to obtain a very great concentration of the bodies in the centrifuged sediment (Fig. 2). When a suspension of intact red cells containing the bodies was

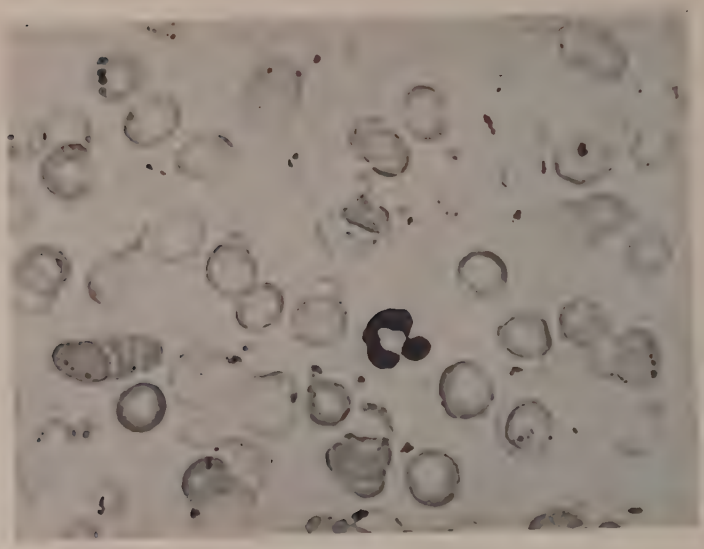

FIG. 1.
Case III. Blood Smear. Giemsa.

FIG. 2.
Case III. Blood laked with distilled water, centrifuged sediment stained with HCl and potassium ferrocyanide. × 460.

placed in a strong electro-magnetic field, the cells with the inclusions were drawn in a linear streak along the sharp edge of the magnet.

In Giemsa stained sections of the spleen, in all 3 cases, minute blue-staining bodies were present within the endothelial cells lining the sinuses. They were found also in reticulo-endothelial cells of lymph-nodes and bone marrow, and in Kuppfer cells, in the 2 fatal cases.

A cocco-bacillus, obtained by culture from the spleen of Case III, proved to be non-pathogenic for splenectomized monkeys, guinea pigs, rats, and mice. Its identity with the intra-corpuscular bodies could not be established, although it showed close morphologic resemblance. Cultures were not agglutinated by the patient's serum.

Blood from Case III, containing about 40% of affected cells, was injected into a splenectomized monkey, and into splenectomized guinea pigs, rabbits, mice, a bartonella-free strain of rats, and into hamsters. The animals failed to develop intra-erythrocytic inclusions, and did not become anemic or otherwise ill.

The essential clinical features in the 3 cases in which the above-described bodies appeared in the peripheral blood *only after splenectomy* were as follows:

Case I was an American-born female of 33. The duration of her illness, which ended fatally, was approximately 4 months; the symptoms were those of a progressive febrile anemia with slight icterus, and enlargement of spleen and liver. Iron, liver extract, and transfusions were ineffective, and the removal of the spleen did not influence the course of the disease. The erythrocytes reached a low count of 900,000 with 3.4 g of hemoglobin. Following splenectomy, platelets and leucocytes were elevated. Reticulocytes ranged from 20 to 26%, and there were many normoblasts. Fragility of the red cells was not altered.

Case II. A white woman, 23 years of age, the duration of whose illness was about 3 months. The clinical course and laboratory findings were similar to those of Case I; continuous spiking fever, slight icterus, enlargement of liver and spleen, and a progressive anemia which failed to respond to transfusions, liver extract, or splenectomy. The R.B.C. fell to 600,000, the Hb to 2.9 g. The reticulocytes were increased. The leucocytes were elevated until shortly before death, when a marked leucopenia rapidly developed. There was a terminal mycotic infection of pharynx, intestine, lungs and spleen.

Case III is an American boy of 19, upon whom splenectomy was performed because of an enlarged spleen and a moderately severe anemia of unknown duration which had failed to improve with the usual anti-anemic treatment. He is at present in fair condition, but his anemia has not been significantly bettered by the operation.

At the last examination, 55% of his erythrocytes contained inclusions.

In spite of intensive study, it has not yet been possible to arrive at a final conclusion as to the nature of these bodies. They are obviously not artefacts, since they can be seen in unstained fresh preparations, and can be stained by a variety of methods. In favor of their parasitic nature is their sharp staining with the Romanowsky stains, and particularly, their very frequent occurrence in diploid or tetraploid form which is strongly suggestive of active division. However, a comparison of the structures with other non-protozoan blood parasites—*Bartonella bacilliformis*,* Hæmobartonellæ, Grahamellæ, and Eperythrozoa—has led to the conclusion that they are not identical with any of these forms. *B. bacilliformis* and *Hæmobartonella muris* do not give an iron reaction when treated with HCl and potassium ferrocyanide.

If they are interpreted as parasitic organisms, one must credit them with the unique property of adsorbing or incorporating into their substance iron from the break-down of hemoglobin. The possibility that the bodies may be non-parasitic, and of the same nature as the siderous granules described by Grüneberg,[1,2] Doniach, Grüneberg, and Pearson,[3]

* Otto and Rezek (*J. Florida Med. Assn.*, 1943, **30**, 62) have recently reported the finding of similar bodies in a splenectomized male of 45, with fatal anemia.

1 Grüneberg, H., *Nature*, 1941, **148**, 114.

2 Grüneberg, H., *The Genetics of the Mouse*, Cambridge University Press, 1943, p. 163.

and by Case,[4] has been considered but no final conclusion reached.

Whatever their nature, the association of these intra-erythrocytic inclusions with febrile anemia resistant to all known anti-anemic treatment is of considerable interest. Further studies are in progress, and will be reported in full elsewhere.

[3] Doniach, I., Grüneberg, H., and Pearson, J. E. G., *J. Path. and Bact.*, 1943, **55**, 23.

[4] Case, R. A. M., *Nature*, 1943, **152**, 599.

14628

Hemoglobin Regeneration in Dogs Receiving a Purified Ration Plus Succinylsulfathiazole.*

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Previous studies have shown that dogs grow quite well and regenerate hemoglobin very rapidly on a sucrose-casein-synthetic vitamin ration when the casein is not highly purified;[1] that blood regeneration on mineralized milk diets is not as rapid even when extra vitamins are added,[2] and that a deficiency of certain of the B-vitamins does not always respond rapidly to administration of the missing vitamin.[3] These variations raised the question as to whether the intestinal flora might not be modified sufficiently by different rations to bring about a decreased production of some unknown factors necessary for the dog.

An attempt was made, therefore, to produce a more consistent and more severe deficiency by adding succinylsulfathiazole to the basal diet and subjecting the dog to severe phlebotomy. Growth and hemoglobin regeneration were equally as good, if not better, in the dogs receiving the drug at levels up to 4% as in the control animals. Since these results are distinctly different from those obtained in rats, we are reporting them very briefly.

Experimental. The basal diet had the following composition: acid washed casein 19%, sucrose 66%, cottonseed oil 11%, salts IV 4%,[4] and was supplemented (on a per kilo body weight per day basis) with 3 drops of haliver oil, 100 γ thiamine and riboflavin, 60 γ pyridoxine, 2 mg nicotinic acid, 500 γ pantothenic acid, and 50 mg choline.†

Eight mongrel litter mates were used, 6 of which (327-332) were put on the experiment immediately after weaning and 2 (325, 326) at the age of 2½ months. They were divided into 4 groups carefully selected on the basis of size and previous weight gains. Dogs 325, 326, 328, and 329 received the basal ration with added succinylsulfathiazole; dogs 330 and 331 were fed the basal ration with succinylsulfathiazole and 2% liver extract (1/20 powder); dog 327, a negative control, received only the basal ration, while dog 332, a positive control, received 2% liver extract (1/20 powder) added to the basal ration. The succinylsulfathiazole was mixed in the ration at a level of 0.5% for 7 weeks, then raised to 1% for 5 weeks and finally increased to 2% for another 3 weeks. Three-cc blood samples

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Wisconsin Alumni Research Foundation.

[1] McKibbin, J. M., Schaefer, A. E., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1942, **145**, 107.

[2] Potter, V. R., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1938, **126**, 155.

[3] McKibbin, J. M., Schaefer, A. E., Frost, D. V., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **142**, 77.

[4] Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, 1935, **109**, 657.

† We are indebted to Merck and Company, Inc., Rahway, N.J., for the generous supply of crystalline B vitamins, to Abbott Laboratories, North Chicago, Ill., for the Haliver Oil, to Wilson Laboratories, Chicago, Ill., for the liver extract, and to Dr. A. D. Welch, Sharp and Dohme, Inc., Glenolden, Pa., for succinylsulfathiazole.

TABLE I.
Hemoglobin Made per Kilo Body Weight per Week, on Different Levels of Succinylsulfathiazole (Sulfasuxidine).

Dog No.	Addition to basal diet	Levels of succinylsulfathiazole 0.5%	1%	2%	Avg for entire experiment
325	Sulfasuxidine		2.29	2.34	
326	"		2.63	3.18	
328	"	1.42	2.14	3.07	1.82
329	"	1.47	2.62	3.31	2.31
330	" + 2% liver extr.	1.92	2.49	3.56	2.28
331	" " " " "	1.59	2.79	1.93	1.81
327	None	1.95	2.41	4.00	2.31
332	2% liver extr.	1.71	1.99	2.68	2.02

were taken weekly from the radial vein for routine hemoglobin, hematocrit, and red blood cell determinations.[5]

Phlebotomy (from the jugular vein) was always heavy (30-40% of the total blood volume at each bleeding) and repeated every other day in order to reduce the hemoglobin level to 6% within one week. The amount of hemoglobin removed was determined as oxyhemoglobin by Evelyn's method; the hemoglobin made was calculated from the difference in total hemoglobin of the dog between one sampling and another, assuming 8% of the body weight as total circulating blood.

For 7 weeks, the dogs were allowed to grow without bleeding. During this period, dogs 325, 326, 328, 329 and 332 received 0.5% succinylsulfathiazole. The drug was then raised to 1% and all the animals bled to anemic levels. Five weeks later, when the hemoglobin level had returned to normal, succinylsulfathiazole was increased to 2% and, after an additional week, all the animals were again made anemic through bleeding. During the recovery period, the level of succinylsulfathiazole was raised to 4% for dogs 328 and 329. After 5 more weeks, these 2 dogs were bled for the third time to a low level of hemoglobin.

Results and Discussion. No differences were observed between the 4 groups of animals, either in rates of growth, hemoglobin regenerated, appetite or general state of health.

Table I shows the amount of hemoglobin made during the different stages of the experiment, calculated weekly per kilo body weight.

In all instances, the hemoglobin level of the dogs returned promptly to normal after cessation of bleeding. (Fig. 1). No plateau in weight was observed. Appetite remained constant throughout. Calculation of the mean cell volume revealed no significant variations at any time of the experiment.

It is evident from these studies that succinylsulfathiazole given at levels up to 4% of the diet fails to affect adversely either growth or blood regeneration in the dog. Since levels as low as 0.5% of the drug are injurious to the rat,[6] one might conclude that the dog does not require fractions such as biotin and "folic acid" which have been shown to be essential to the rat through the use of sulfa drugs. However, it is more likely that succinylsulfathiazole does not modify the intestinal flora sufficiently to produce a deficiency of these additional factors in the dog. It is well known that sulfapyridine produces additional deficiencies in dogs suffering from a nicotinic acid deficiency.[7] Preliminary work indicates that the feeding of sulfathiazole to nicotinic acid-deficient dogs produces similar conditions, but that succinylsulfathiazole has no adverse effect.‡

Although the casein used in these studies was the same as that used in our studies with

[5] Spector, H., Maass, A. R., Michaud, L., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1943, **150**, 75.

[6] Ransone, B., and Elvehjem, C. A., *J. Biol. Chem.*, 1943, **151**, 109.

[7] Schaefer, A. E., McKibbin, J. M., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **144**, 679.

‡ Krehl, W. A., and Elvehjem, C. A., unpublished data.

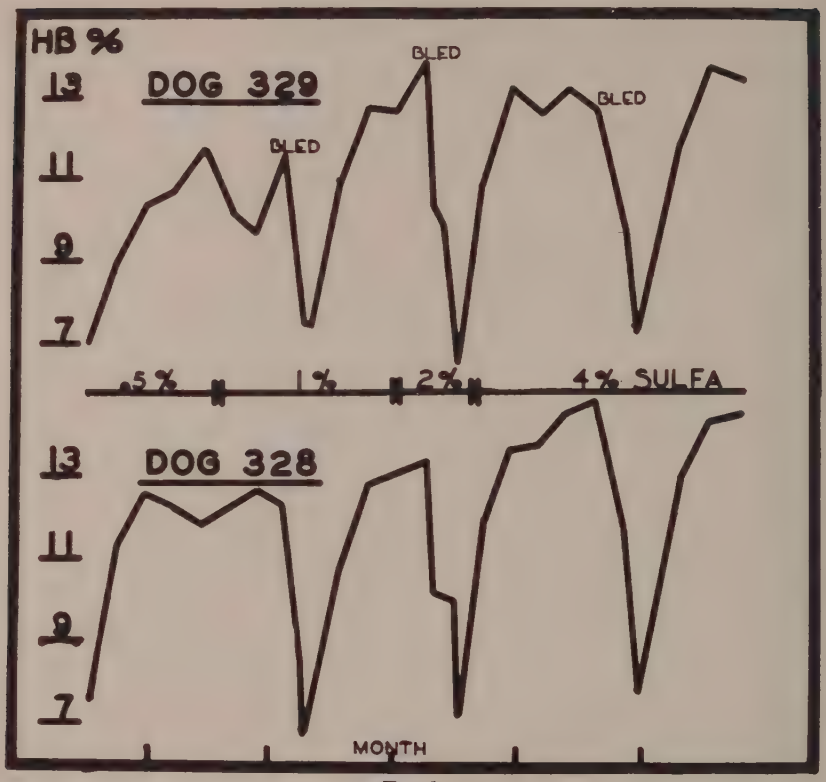

FIG. 1.

Typical hemoglobin picture of 2 dogs receiving varying levels of succinylsulfathiazole in their ration.

rats, it is possible that a deficiency would have been produced if the casein had been more highly purified. Lambooy and Nasset[8] have recently produced definite deficiency symptoms in young dogs by feeding a synthetic diet containing purified casein and sucrose but no sulfa drug. However, their ration contained added inositol and *p*-aminobenzoic acid which may have altered the intestinal flora. Smith[9] has also reported an anemia in dogs on a synthetic ration but again inositol and *p*-aminobenzoic acid were added. Thus, intestinal synthesis may be as important in dogs as in rats but the flora is not altered as readily by succinylsulfathiazole.

Summary. Succinylsulfathiazole was fed to dogs at levels varying from 0.5% to 4% of the ration in an attempt to modify the intestinal flora and cause a deficiency of factors possibly synthesized in the digestive tract.

No differences were observed between the animals receiving the drug and those serving as controls. Hemoglobin per 100 g of blood, total hemoglobin in the dogs, red blood cells, mean cell volume, food consumption, growth increase and general state of health were taken into consideration.

[8] Lambooy, J. P., and Nasset, E. S., *J. Nutrition*, 1943, **26**, 293.

[9] Smith, Susan Gower, *Federation Proc.*, 1944, **3**, 96.

14629

Nutritional Effects on the Development and Atrophy of the Thymus.

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In inanition (Jonson)[1] and in Vitamin B complex deficiency (Cramer, Drew and Mottram)[2] low thymus weights are found. Griffith and Wade[3] have reported a graded response of thymus weights when increasing supplements of choline are added to "choline deficient" diets. Christensen and Griffith[4] suggest that "choline probably represents the factor required for maintenance of the thymus."

The thymus data recorded below taken from a larger series of experiments dealing with various phases of the behavior of rats on single deficiencies of B factors (Berg and Zucker)[5] throw further light on thymus growth and thymus atrophy.

Results. The basal diet contained 26.7% of casein. The salt mixture was essentially that of Wesson. The fat soluble vitamins were given as a solution of cod liver oil carotene and wheat germ oil in cotton seed oil, 1 mg of niacin and 10 mg of choline hydrochloride were given daily to each rat. The B factor variables are given in Table 1, columns 1-4. Series A represents controls. In each of the experiments of Series B one of the 4 factors under investigation was completely omitted while Series C represents partial deficiencies. The next 3 columns (5, 6, and 7) give respectively initial and final mean body weights and the mean observed thymus weight. In columns 8 and 9 are found the thymus weights as found in stock diet animals corresponding in body weight to the initial and final mean body weights of the experimental groups. These values were derived from a long series of observations covering the experimentally useful age and body weight range (Stoerk)[6]. They are used as a base line for judging thymus growth during the 4 weeks of experiment (column 9) and the deficit in thymus weight (column 10) shown by the experimental animals as compared with normal animals of similar body weight. The data of column 8 and 9 are quite comparable to those used by Christensen and Griffith for like purposes. Column 12 designates by plus and minus signs the degree of atrophic changes seen in sections.

No attempt is made to put an exacting interpretation on the numerical values but the following indications seem reasonable.

In experiment A1 (positive control) the body weights and thymuses grow as in stock diet animals. From 4 weeks to 8 weeks of age body weight increases from 62 to 150 g and the thymus weight increases by 146 mg. A2 may be considered a negative control in which all four B factors under investigation were omitted from the diet. There is a body weight loss of 11 g and the thymus weight is 248 mg less than that found for normal body weights corresponding to the initial body weight.

With total deficiency of riboflavin and of pantothenic acid (B_1 and B_2) body growth is fair and there is no marked loss of thymus tissue (although the mean values for thymus increase are negative) but the observed thymus weight falls distinctly below that of normal animals corresponding to the final body weights.

With the total thiamin or pyridoxin deficiency (B_3 and B_4) there is also no net loss

1 Jonson, A., *Arch. f. mikr. Anat.*, 1909, **73**, 390.

2 Cramer, W., Drew, A. H., and Mottram, J. C., *Lancet*, 1921, **201**, 1202.

3 Griffith, W. H., and Wade, N. J., *J. B. C.*, 1939, **131**, 567.

4 Christensen, K., and Griffith, W. H., *Endocr.*, 1942, **30**, 575.

5 Berg, B. N., and Zucker, T. F., Abstracts 107th A.C.S. Meeting, Cleveland, Ohio, April, 1944.

6 Stoerk, Herbert C., *Endocr.*, 1944, **34**, 329, and data to be published.

in body weight but the thymuses decidedly fall below the weight at the beginning of the experiments. The deficit below the thymus weights expected for final body weights is not much larger because there was little body growth.

While in all the experiments under B_1 there was a net gain in body weight, the growth was not necessarily continuous. Effects on the thymus weight might therefore be looked upon as inanition phenomena rather than specific effects of the particular vitamin deficiencies. In series C partial deficiencies were produced by giving 1/20 to 1/50 of the approximate requirement of the particular factor under investigation. In these experiments net growth was somewhat better and was in every animal continuous, *i.e.* no weight losses occurred during the 4 weeks of experiment.

In the case of partial deficiency of riboflavin and pantothenic acid there was increase in thymus weight to about half the extent of the thymus increase in optimally growing rats. Still the thymus did not reach weights normal for the final body weights achieved. With partial thiamin deficiency the thymus weight did not deviate much (—26 mg) from that corresponding to the initial body weight. In marked contrast to this the animals with partial pyridoxin deficiency lost an estimated 153 mg of their initial thymus weight while there was better body growth than in the corresponding thiamin deficient rats.

Summary. 1. In otherwise adequate diets complete or partial deficiency of riboflavin, pantothenic acid, thiamin or pyridoxin depresses the thymus weight of female albino rats below that of normally grown controls of corresponding body weight. 2. With complete deficiency of any one of the four factors mentioned, there is mild to marked atrophy of the thymus which may however be referable to losses in body weight. 3. On a diet partially deficient in thiamin which produces small but continuous body weight increases, thymus growth may be arrested for the four weeks duration of the experiment while under similar conditions partial pyridoxin deficiency produces marked atrophy.

The observations all refer to an age range before "age involution" sets in. The atrophic

TABLE I.

		1	2	3	4	5	6	7	8	9	10	11	12		
		B supplements γ/day				Mean body wt, g		Mean observed thymus wt, mg	Mg thymus corresponding to body wt		Thymus inc. over initial	Deficit below expected final thymus wt.	Histol. signs of atrophy	No. of animals
		Panto	Ribo	Thia	Pyri	Initial	Final		Initial	Final				
A	1	200	80	40	50	62	150	536	290	536	+146	0	—	12
	2	0	0	0	0	58	47	12	260	240	—248	—228	+++	10
B	1	0	80	40	50	68	105	301	320	420	— 19	—119	—	3
	2	200	0	40	50	63	103	256	300	410	— 44	—154	—	7
	3	200	80	0	50	67	85	128	310	360	—182	—232	+ to ++	6
	4	200	80	40	0	66	88	77	310	370	—233	—293	++ to +++	4
C	1	5	80	40	50	60	114	361	280	440	+ 81	— 79	—	8
	2	200	2	40	50	61	105	354	290	420	+ 64	— 66	—	11
	3	200	80	2	50	59	94	234	260	390	— 26	—156	—	7
	4	200	80	40	1	62	103	127	290	410	—153	—273	+ to ++	7

changes would be classed under the term "accidental involution." They occurred in rats each of which received 10 mg of choline hydrochloride per day.

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Effect of the Endotoxin of *Shigella paradysenteriae* on Pregnancy in Rabbits.

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We have previously reported that the endotoxins of certain gram-negative bacteria, when injected into mice at middle and late pregnancy, induce decidua-placental hemorrhage which often leads to abortion or resorption of the embryos.[1] This hemorrhage induction was considered to be analogous to that induced in implanted mouse tumors by the endotoxins of essentially all gram-negative bacteria. The present study was undertaken to ascertain the effect of such endotoxins on pregnancy in rabbits.

Methods. The acetone precipitate of the residue of dialyzed whole cultures of *Shigella paradysenteriæ* (Flexner) was chosen as containing a representative O antigen of Boivin-type, the principal endotoxin of most gram-negative bacteria. Methods of culture, preparation and standardization are described elsewhere.[2]

The experimental procedure consisted of the intraperitoneal injection into rabbits at various stages of pregnancy of an arbitrarily selected sublethal dose of the Flexner extract. This dose (16 mg) of relatively unpurified material was equivalent to 2 LD_{50} for 20 g white mice or about one-fifth of that lethal to rabbits of 3-4 kg. This high sensitivity of rabbits to the endotoxin of gram-negative bacteria has been noted by other investigators.[3]

The female Rockland rabbits used in this study were multiply mated, a procedure assuring 80-100% true pregnancies. Rabbits so mated received intraperitoneal injections of the endotoxin-containing extract at one of the following stages of pregnancy: (1) 48 hours after mating, *i.e.*, after ovulation and fertilization, but before uterine implantation, (2) 6-7 days after mating, *i.e.*, during the implantation period, (3) 11-12 days after mating, *i.e.*, post-implantation and during development of placental structures, (4) 15-16 days and (5) 19-21 days after mating, *i.e.*, during active growth of embryos and placental tissues, and (6) 25-26 days after mating, *i.e.*, approaching term. Following injection, the animals were observed daily for vaginal bleeding or for evidence of abortion. They were either allowed to proceed to term, or were sacrificed 5 to 10 days after injection, when post-mortem examination was made of the reproductive organs and embryonic tissues. If the embryos and associated membranes appeared to be normal in such sacrificed animals, it was assumed that pregnancy had not been impaired or interrupted.

Results. Of 6 animals injected 48 hours after mating, 5 failed to show further signs of pregnancy. The sixth delivered a normal litter. Of the animals injected at the 6-7-day stage, only one out of 7 underwent interruption of pregnancy. At the 11-12-day stage, 2 out of 4 animals had litters. In the 4 animals at the 15-16-day stage and in the 7 animals at the 19-21-day stage there was uniform interruption of pregnancy although 2 animals of the latter group died within 4 days after injection. At the late stage of 25-26 days 2 out of 9 rabbits delivered normal litters. Among the remaining 7, 3 aborted and 4 died, possibly

[1] Zahl, Paul A., and Bjerknes, C., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 329.

[2] Zahl, Paul A., Hutner, S. H., and Cooper, F. S., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 48; 1943, **54**, 187; Zahl, Paul A., and Hutner, S. H., *Am. J. Hyg.*, 1944, **39**, 189.

[3] Grasset, E., Zoutendyke, A., and Schaafsma, A., *Brit. J. Exp. Path.*, 1935, **16**, 454; Boivin, A., and Mesrobeanu, L., *C. R. Soc. Biol.*, 1935, **118**, 614.

from toxemia arising from an inability to evacuate dead or injured fetuses. Rabbits injected at stages beyond 15 days often displayed vaginal bleeding and abortion of embryos. In other animals, at post-mortem, dead or resorbing embryos were often found as nodular masses of tissue in the uterine horns. The habit of some rabbits to eat fetuses aborted at night made precise observation in this connection uncertain. A relatively uniform sequel to the injection, usually within the first 24 hours, was profuse diarrhea.

We have been unable to identify the precise site of the hemorrhage but have generally supposed it to occur in the decidua-placental tissues. The aborted fetuses themselves showed no sign of purpura or other evidences of hemorrhage, and the normal rabbit placenta is so highly vascular a tissue as to render observation of induced hemorrhage difficult.

As in the mouse, we observed no sign of hemorrhage or purpura in the ovary and its component tissues, in the adrenals, nor in the normal and pseudo-pregnant uterus. Although none of the rabbits used in this study were rebred, mice in which abortion had been induced by the injection of the endotoxin have consistently given birth to normal litters following rebreeding. This would seem to indicate that the dose of endotoxin employed to induce abortion had no permanently injurious effect on the tissues associated with reproduction.

Discussion. The assumption that decidual and placental capillaries, like those of rapidly growing tumors, have a much lower threshold of damage than most other body tissues to vascular poisons of the type found in gram-negative bacteria seems valid as an explanation of the effect of such toxins on middle and late pregnancy when vascular growth is rapid and extensive in the accessory embryonic structures, but it does not explain the interruption of pregnancy during the earlier stages, before implantation or before extensive vascularization has occurred. An alternative explanation for the latter effect may be derived from the thermal changes which occur in the rabbit almost immediately following injection of the endotoxin. The fever curve in Fig. 1 is typical of the thermal changes which occur in the rabbit given the dose of endotoxin by the route of injection employed in this study. Within a few hours after injection the rabbit exhibits a hyperthermia, usually attaining a maximum of 41-42°C within 3 to 6 hours, followed by a slow return to normal during the next 20 hours. That such fever in rabbits is produced by the endotoxins of other gram-negative bacteria is supported by a considerable body of work, representative reports of which are those by Welch *et al.*[4] and by Co Tui.[5]

Cameron[6] has shown that fever (42-43°C) induced by partial submersion in warm water for 20 minutes will interrupt pregnancy in rabbits mated 72-80 hours previously, although, as pointed out by Cameron, artificial fever is generally regarded clinically as a safe form of therapy in middle or late pregnancy. Cameron interprets this as an indication that the rabbit blastocyst has low viability at the fever temperatures indicated. In view of Cameron's findings, it seems reasonable to suggest in connection with the data reported here that the fever induced by the bacterial endotoxin caused death to the blastocyst, thus accounting for the interruption of pregnancy during the pre-implantation stage.

The somewhat unexpected failure of the endotoxin to interrupt pregnancy at the 6-7-day stage could yield to the corollary explanation that the embryo at this stage has overcome its thermosensitivity, and that there has been as yet no extensive vascular infiltration between placental and decidual tissues on which the hemorrhagic factor of the endotoxin could operate. Even if some vascular damage did occur in the decidual tissues, it would not necessarily result in atrophic conditions fatal to the embryos. However, some days after the 6-7-day stage, when vascularization has become definite and well established, it appears that the endotoxin gives rise (as denoted by frequent instances of vaginal bleeding) to hemorrhage in the rapidly growing decidua-placental tissues, causing severe vascular im-

[4] Welch, H., Calvery, H. O., McClosky, W. T., and Price, C. W., *J. Am. Pharm. Assn.*, 1943, **32**, 65.

[5] Co Tui, *J. Lab. and Clin. Med.*, 1944, **29**, 58.

[6] Cameron, J. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 76.

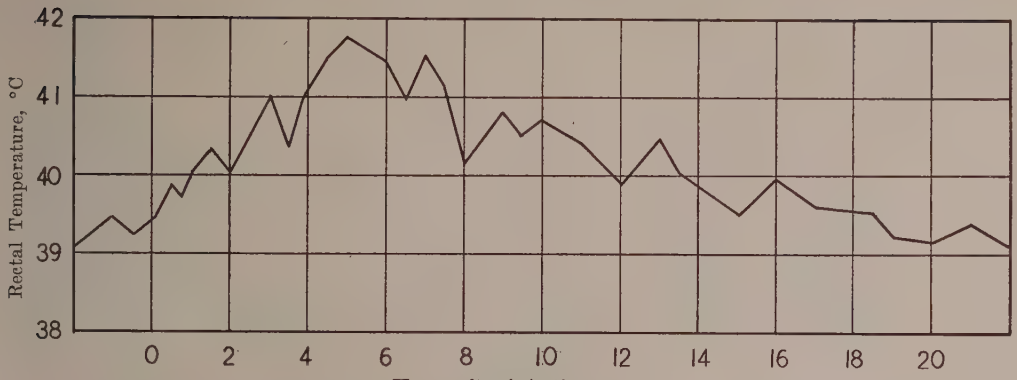

FIG. 1.

Typical temperature chart for female rabbit receiving intraperitoneal injection of 1/5 MLD of the endotoxin of *Shigella paradysenteriæ* (Flexner).

pairment, and leading to resorption or abortion.

Sanarelli[7] states that cholera in pregnant women almost always leads to abortion, and that vibrios injected into pregnant rabbits also give rise to abortion even in the absence of an active infection. Jennings and Mathieson[8] report that abortion or premature labor occurs in 40-60% of pregnant women with typhoid fever infection. They note further that when typhoid infection occurs in the early months of pregnancy, abortion is accompanied by severe hemorrhage. It is to be pointed out that both *Vibrio comma (choleræ)* and organisms of the typhoid group are gram-negative bacteria whose principal toxic factor is the Boivin-type of endotoxin common to the organisms used by us and, indeed, to most gram-negative bacteria. In previous surveys[9] this type of endotoxin has been shown, almost without exception, to be capable of inducing hemorrhage in implanted mouse tumors.

Summary. Relatively large sublethal doses of the endotoxin-containing extract of *Shigella paradysenteriæ* (Flexner) were injected intraperitoneally into rabbits at the 2, 6-7, 11-12, 15-16, 19-21, and 25-26-day stages of pregnancy. Pregnancy at the 2-day stage was interrupted following such injection, possibly because of a low viability of the rabbit blastocyst to the marked hyperthermia which develops following the injection. The failure of the endotoxin to interrupt pregnancy at the 6-7-day stage is discussed. At stages between the 11th and 26th day, pregnancy was interrupted, accompanied often by vaginal bleeding and abortion which resulted presumably from the hemorrhagic action of the endotoxin on fragile capillaries of the decidua-placental tissues.

This decidua-placental hemorrhage in rabbits is construed to be an action physiologically analogous to the hemorrhage induced by the endotoxins of gram-negative bacteria in implanted mouse tumors.

7 Sanarelli, G., *Ann. Inst. Pasteur*, 1924, **38**, 11.

8 Jennings, A. F., and Mathieson, D. R., *Practice of Medicine*, 1940, W. F. Prior Co., Hagerstown, Md.

9 Zahl, Paul A., Hutner, S. H., Spitz, S., Sugiura, K., and Cooper, F. S., *Am. J. Hyg.*, 1942, **36**, 224; Hutner, S. H., and Zahl, Paul A., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 364.

14631

Temperature Factors in the Action of Certain Bacterial Endotoxins.

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It has been previously reported that the endotoxin of *Shigella paradysenteriae* (Flexner) administered intraperitoneally to gravid rabbits induces a hyperthermia which develops at the same time as decidua-placental hemorrhage.[1] The same endotoxin when injected into mice induces hemorrhage both in implanted tumors and in decidua-placental tissues.[2] The concurrence of thermal and histopathological effects in the rabbit following injection of the Flexner endotoxin suggested a study of this relationship in the mouse.

Experimental. Record was kept of rectal temperatures following the intraperitoneal injection of the endotoxin-containing acetone precipitate of the residue of dialyzed whole cultures of *Shigella paradysenteriae* (Flexner) into (1) mice bearing 7-day-old implants of mouse sarcoma 180, and into (2) mice at approximately the 15th day of pregnancy.* Endotoxin-containing extracts of *Salmonella typhimurium* and *Rhodospirillum rubrum* were similarly employed. These 3 organisms, 2 closely related and the third taxonomically remote from the other 2, were chosen as representative gram-negative bacteria. Methods of culture preparation and standardization of these materials are described elsewhere.[3] An arbitrary dose of ¼ LD_{50} was selected for testing the 3 preparations. In addition, doses of 1/200, 1/100, 1/50, 1/20, 1/10, and 2 LD_{50} of the Flexner extract were injected in tumor-bearing mice, and changes in rectal temperature recorded (Fig. 1).

Changes induced by exposing tumor-bearing mice and 15-day-pregnant mice for a sustained period to external heat and to cold were also investigated. An incubator maintained at 37°C constituted the heat chamber, while the cold chamber was a refrigerator kept at 4-6° C. Rectal temperature for mice kept in the incubator for 4 hours ranged between 39° and 41° C, and for mice kept in the refrigerator for 12 hours the rectal temperature range was 34-36° C.

The 200 Rockland mice used in this study were of a standardized inbred strain tested at regular intervals for freedom from intestinal infections.

Results and Discussion. Within a short time after the injection of ¼ LD_{50} of the endotoxins, a marked hypothermia developed, the rectal temperature often falling below 30° C (Fig. 1). Closely associated in time with the abrupt drop in temperature was the appearance of tumor-hemorrhage in tumor-bearing mice, and vaginal bleeding and often abortion in pregnant mice. There was no observed difference in the action of the endotoxin-containing preparations of *Shigella, Salmonella* or *Rhodospirillum*. Neither of the hemorrhage effects was evident in the control mice exposed to low or to high external temperatures.

Following the administration of 2 LD_{50}, an abrupt hypothermia developed often reaching a minimum of 24° C, followed by death within 5 to 24 hours from the time of injection. Doses below 1/10 LD_{50} did not appear to induce significant changes in rectal temperature (Fig. 1). Severe diarrhea was a uniform consequence of the injection of the 1/10, ¼ and 2 LD_{50} doses.

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1 Zahl, Paul A., and Bjerknes, Clara, PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 153.

2 Zahl, Paul A., and Bjerknes, Clara, PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 329.

* It is generally agreed that the toxic effects of extracts of *Shigella paradysenteriæ* (Flexner) and of *Salmonella typhimurium* are referable solely to the endotoxin whose pattern of pathogenesis is characteristic of the endotoxins of most gram-negative bacteria. In view of this, we have for convenience chosen to designate our relatively unpurified preparations as endotoxins.

3 Zahl, Paul A., Hutner, S. H., and Cooper, F. S., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 48; 1943, **54**, 187; Zahl, Paul A., and Hutner, S. H., *Am. J. Hyg.*, 1944, **39**, 189.

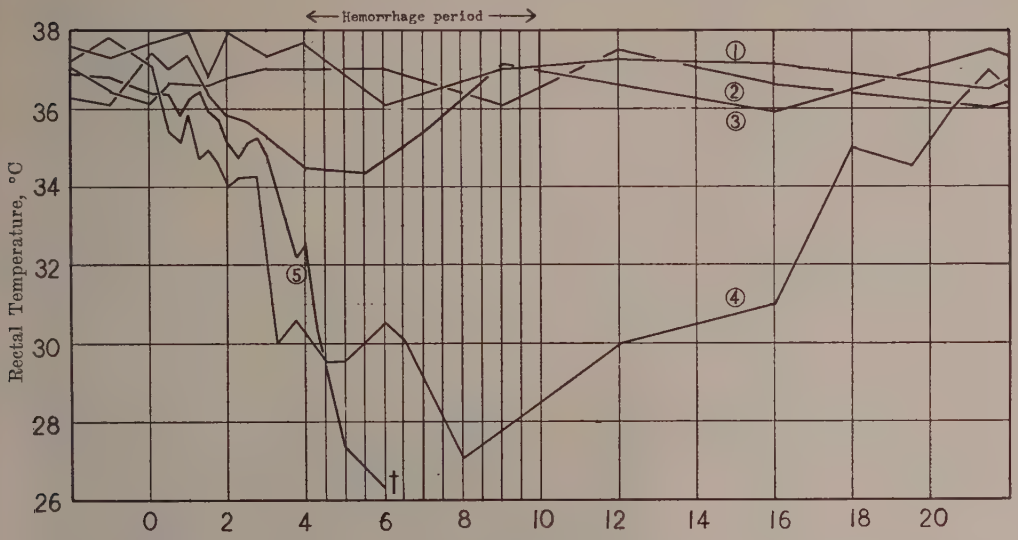

FIG. 1.

Typical temperature curves for tumor-bearing mice receiving various intraperitoneally injected doses of the endotoxin of *Shigella paradysenteriæ* (Flexner). (1)—1/100 LD_{50}; (2)—1/50 LD_{50}; (3)—1/10 LD_{50}; (4)—1/4 LD_{50}; (5)—2 LD_{50}. † = death.

There appears to be no direct causal relationship between the endotoxin-induced hypothermia in mice and the parallel histopathological changes. This view is supported by a body of data to the effect that (1) deciduaplacental hemorrhage in rabbits is paralleled by hyperthermia, whereas in mice it is paralleled by hypothermia, (2) doses between 1/10 and 1/200 LD_{50} in mice regularly induce tumor-hemorrhage,[3] but without appreciable concurrent thermal changes, (3) the hypothermia induced by refrigeration induces neither tumor- nor decidual-placental hemorrhage.

A number of workers[4–6] have reported marked hypothermia in guinea pigs, following injection of killed suspensions of various gram-negative bacteria. Feldman and Gellhorn[7] found that in rats a stimulation of the vago-insulin system (a portion of the parasympathetic) is followed by decrease in body temperature and blood sugar, and that stimulation of the sympathetico-adrenal system leads to rise in both body temperature and blood sugar. From the results of injecting typhoid-paratyphoid vaccine into normal, into vagotomized, and into adreno-demedullated rats, they concluded that both systems were simultaneously stimulated, with stimulation of the sympathetico-adrenal system predominating.

Applying this explanation, it would appear that in the mouse and the guinea pig the endotoxin of gram-negative bacteria stimulates predominantly the vago-insulin system, while in the rat,[7] the rabbit,[1] the cat,[8] and man,[8] action of the sympathetico-adrenal system predominates but with evidence often of a rhythm in dominance of first one system and then the other. This hypothesis appears to be in accord with the studies of Delafield and collaborators[9] who observed marked initial hyper-

[4] Sanarelli, J., *Ann. Inst. Pasteur*, 1893, **7**, 225.

[5] Pfeifer, R., *Z. f. Hyg.*, 1892, **11**, 393.

[6] Olitsky, L., Avering, S., and Koch, P. K., *J. Immunol.*, 1942, **45**, 237.

[7] Feldman, J., and Gellhorn, E., *Endocrinology*, 1941, **29**, 141.

[8] Ranson, S. W., Jr., *Arch. Int. Med.*, 1938, **61**, 285; Ranson, S. W., Jr., Clark, C., and Magoun, H. W., *J. Lab. and Clin. Med.*, 1939, **25**, 160.

[9] Delafield, M. E., *J. Path. and Bact.*, 1932, **35**, 53; Cameron, G. R., Delafield, M. E., and Wilson, G., *Idem.*, 1940, **51**, 223.

glycemia followed by hypoglycemia in rabbits injected with either the crude or purified endotoxins of *Salmonella typhimurium* and many other gram-negative organisms. These changes did not occur following the injection of extracts of gram-positive organisms.

It has generally been supposed that the endotoxins of gram-negative bacteria are direct vascular poisons affecting the capillary endothelium.[10] However, in view of the above considerations, an alternative explanation would refer the locus of damage to the innervation of the capillaries rather than to the capillary walls directly. This point of view would link the otherwise apparently unrelated thermal and histopathological effects of the endotoxins of gram-negative bacteria.

The character of the end results, *viz.*, (1) disturbances in temperature regulation, (2) disturbances in carbohydrate metabolism, and (3) various hemorrhagic phenomena, may point also to possible damage localized at a high level of the nervous system. This level may be at the hypothalamus, in view of its recognized role in the regulation of temperature and carbohydrate metabolism. In addition, it has been repeatedly observed[11] that lesions of the hypothalamus induce marked hemorrhagic conditions in the gastro-intestinal mucosa, a tissue notably sensitive to damage by the endotoxins of gram-negative bacteria.

The selective character of endotoxin-induced damage to capillaries in implanted tumors, in decidua-placental tissues, and in the gastro-intestinal mucosa can be as well explained by assuming a higher sensitivity to hypothalamic or to direct stimulation of the local innervation of these tissues, as by assuming a high sensitivity of the endothelial walls to direct toxic action. The long-known localization of colloid azo-dyes in the types of lesion under discussion is not in conflict with such a thesis. The greater permeability to large colloid particles on the part of capillary walls in these tissues may rather reflect the condition of the innervation controlling the tonus of the capillary walls.

Summary. The endotoxin of *Shigella paradysenteriae* (Flexner) induced in mice a marked hypothermia which developed at the same time as hemorrhage in implanted tumors and in decidua-placental tissues. Similar effects were observed for the endotoxins of *Salmonella typhimurium* and *Rhodospirillum rubrum*.

Hypothermia and hyperthermia induced in tumor-bearing and pregnant mice by exposure to high and low temperature air baths were not paralleled by observable hemorrhagic effects.

It is concluded that the endotoxin-induced hypothermia in mice and the previously reported endotoxin-induced hyperthermia in rabbits bear no direct causal relationship to the histopathological effects which develop in parallel to these thermal changes. An hypothesis based on the assumption that the endotoxins of gram-negative bacteria act as possible neural poisons rather than as exclusively vascular poisons is discussed.

[10] For appropriate literature citations, *vide*: Zahl, Paul A., Hutner, S. H., and Cooper, F. S., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 137.

[11] Fulton, J. F., *Physiology of the Nervous System*, Oxford University Press, N.Y., 1943, p. 241.

14632 P

Immunologic Relations of the Psittacosis-Lymphogranuloma Group of Viral Agents.*

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Investigations in recent years have revealed a group relationship of viral agents,[1-6] isolated from various sources (man, birds, animals), and morphologically similar to the viruses of psittacosis and lymphogranuloma venereum.[7] Interest in this group has been stimulated through recognition of the frequency of infections in man with several of these agents.[8,9] The need for laboratory procedures which might aid in the recognition and study of these viruses has become increasingly evident.

Serological reactions have been of little value in differentiation and identification of the members of this group. Complement fixation tests, although of value in detecting infection by a member of the group,[9, 10] have indicated a broad antigenic similarity and have failed in general to differentiate sharply between the various agents.[11] The serum neutralization test, which has been perhaps of greatest value in identifying other viruses, was not applicable here because in general it has been the experience of investigators that infection in man and animals,[12] or artificial immunization with these agents,[3,13] does not produce a serum satisfactory for studying the antigenic relationships within the group by the neutralization test.

We have previously reported[13] that a neutralizing and protective antiserum of relatively high titer can be obtained against the Chicago strain of mouse pneumonitis,[14] a member of this group, by repeated intraperitoneal injections of chickens with infected mouse lungs. By similar methods we have more recently produced antiserums against the viruses of meningopneumonitis[15] and lymphogranuloma venereum. In the case of the latter virus we were unable to produce a neutralizing antiserum by injection of infected mouse brain but were successful when infected yolk sac was used. This report records our investigations to date on the antigenic relations of these viruses as studied by means of the serum neutralization test.

The tests were run by mixing 0.3-cc quantities of serial 10-fold dilutions of viral suspensions with equal volumes of serum. A series of virus dilutions mixed with normal chicken serum was always included for purposes of comparison. Following incubation at 22°C for one hour, groups of 6 mice (10 g)

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† Holder of the Jessie Horton Koessler Fellowship of the Institute of Medicine of Chicago, 1943-44.

1 Beck, M. D., Eaton, M. D., and O'Donnell, R., *J. Exp. Med.*, 1942, **79**, 65.

2 Pinkerton, N., and Moragues, V., *J. Exp. Med.*, 1942, **75**, 575.

3 Francis, T., Jr., and Magill, T. P., *J. Exp. Med.*, 1938, **68**, 147.

4 Rake, G., Shaffer, M. F., and Thygeson, P., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 545.

5 Thomas, L., and Kolb, E. M., Proc. Soc. Exp. Biol. and Med., 1943, **54**, 172.

6 Nigg, C., and Eaton, M. D., *J. Exp. Med.*, 1944, **79**, 497.

7 Rake, G., and Jones, H. P., *J. Exp. Med.*, 1942, **75**, 323.

8 Meyer, K. F., *Medicine*, 1942, **21**, 175.

9 Smadel, J. E., *J. Clin. Inv.*, 1943, **22**, 57.

10 Eaton, M. D., and Corey, M., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 165.

11 Rake, G., Eaton, M. D., and Shaffer, M. F., Proc. Soc. Exp. Biol. and Med., 1941, **48**, 528.

12 Eaton, M. D., Beck, M. D., and Pearson, H. E., *J. Exp. Med.*, 1941, **73**, 641.

13 Hilleman, M. R., and Gordon, F. B., *Science*, 1943, **98**, 347.

14 Karr, H. V., *J. Infect. Dis.*, 1943, **72**, 108.

15 Hilleman, M. R., and Gordon, F. B., *J. Bact.*, 1944, **47**, 58 (Abstract).

TABLE I.
Representative Serum Neutralization Test with Meningopneumonitis (MP-F97) Virus.

Serums	Final dilutions of virus									Mean Infectivity Scores
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	
Normal	5.0*	5.0	5.0	4.5	3.6	1.2	1.0	0.8	0.0	2.9
Antimeningopneumonitis (MP-F97)	5.0	5.0	2.8	1.3	1.0	0.8	0.0	0.0	0.0	1.8
Difference of mean infectivity scores (Numerical value for neutralizing ability of meningopneumonitis antiserum)										1.1

* Numbers represent average infectivity scores of 6 mice.

TABLE II.
Serum Neutralization Tests in Mice with the Psittacosis-lymphogranuloma Group of Viruses Using Chicken Antiserums.

Virus	Antiserums against: Meningopneumonitis (MP-F97)	Mouse pneumonitis (Chicago)	Lymphogranuloma venereum
Meningopneumonitis (MP-F97)	+	0	
Ornithosis (207-Meyer)	+	0	
Mouse pneumonitis (Chicago)	0	+	0
Mouse pneumonitis (Atherton)	0	+	
Feline pneumonitis (Baker)	0	0	
Lymphogranuloma venereum	0	0	+

\+ = neutralization.
0 = absence of neutralization.

were inoculated intranasally under light ether anesthesia with 0.03 cc amounts of each mixture. On the 10th day all mice yet alive were autopsied and the extent of lung consolidation noted. Table I shows the results obtained in a representative titration, that of meningopneumonitis virus with its homologous antiserum. A numerical value for the infectivity of each mixture is obtained by averaging the infectivity scores of the individual mice, determined by the method of Horsfall.[16] By averaging the scores of a single serum a mean infectivity score is derived. By subtracting this mean infectivity score for immune serum from that of the normal, a figure is derived which gives a numerical value (1.1, Table I) to the neutralizing capacity of the serum for any given virus. This is admittedly a crude method but the single figure has the advantage of allowing a simple comparison between two serums or between the neutralizing capacity of a single serum for different viruses.

Using the 3 antiserums prepared in chickens cross neutralization tests were run and other members of this group of agents were tested in the combinations indicated in Table II. It will be seen that meningopneumonitis antiserum neutralized ornithosis virus as well as the homologous strain, but none of the other viruses against which it was tested. Antiserum produced against the Chicago strain of mouse pneumonitis neutralized not only the homologous strain but also the Atherton II strain.[6] Lymphogranuloma venereum antiserum failed to neutralize mouse pneumonitis (Chicago) the only heterologous virus tested to date.

It is noteworthy that no partial neutralizations occurred with these chicken antiserums. In the 2 cases of heterologous neutralization the serums neutralized to full titer, *e.g.*, the difference of mean infectivity scores for meningopneumonitis antiserum against meningopneumonitis virus was 1.1 (Table I); against ornithosis virus it was 1.3. The 2 corresponding figures for the mouse pneumonitis antiserum were 2.0 for the homologous strain (Chicago) and 1.8 for the heterologous (Atherton). Likewise where neutralization did not occur the figures were 0.0 or nearly so, indicating no difference between immune and normal serum. Our tests therefore indicate complete antigenic similarity or dissimilarity insofar as the neutralizing antibody is con-

[16] Horsfall, F. L., *J. Exp. Med.*, 1939, **70**, 209.

cerned. Serums of pigeons infected with psittacosis have likewise been reported as being highly specific.[17,18] Chickens have been found advantageous for the preparation of antiserums against a variety of substances (See reference 13).

The relations within this group as revealed by serum neutralization tests are in agreement with the relations established on other bases by other authors.[1,2,5,6,11,19] Our experiments suggest that neutralization tests with this type of serums is a more satisfactory method for studying the antigenic relations of this group of agents than those methods heretofore described.

[17] Eddie, B., and Francis, T., Jr., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 291.

[18] Smadel, J. E., Wertman, K., and Reagan, R. L., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 70.

[19] Hamre, D. M., and Rake, G., *J. Bact.*, 1944, **47**, 312.

14633 P

Comparison of the Acid Humoral Intermediation of Stimulation in Respiratory and Non-Respiratory Muscles.*

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The strength of contraction of the rectus abdominus muscle of the frog immersed for varying periods (15-60 seconds) in weak acetylcholine solution (about 1 part in 500,000 parts of Ringer's solution, depending on the sensitivity of the preparation) was found to increase with the hydrogen ion concentration of the environment. Such effects occurred within a range of pH 7.5 to pH 5.0. Lactic, phosphoric, and carbonic acids produced comparable effects. These results agree with those previously reported in the rectus abdominus.[1, 2] However, when the sartorius muscle was used it was found to respond with a weaker contraction under increasing hydrogen ion concentration of the environment. This unexpected finding led us to make comparative observations on a number of muscles of the frog, turtle and alligator. The rectus abdominus, mylohyoid and geniohyoid of the frog, mylohyoid of the turtle, and diaphragm of the alligator, muscles which contribute to the respiratory act, were found to show a progressively increasing strength of contraction with an increasing cH of the acetylcholine-containing environment, while the sartorius, peroneus longus and gracilis minor of the frog, muscles possessing primarily a locomotor function, showed either a consistent depression or an initial augmentation followed by an early depression.

This differential effect of acid on these two groups of muscles is of interest in suggesting, for the lower forms at least, that the control of breathing is not strictly a central reflex phenomenon but is supported by a peripheral motor adjustment as well. The chemical (acid) control of respiration may thus be extended to the respiratory muscles as well as to the carotid body and the intra-cranial portions of chemically sensitive control centers. How the differential humoral response of respiratory and non-respiratory muscle to cH has been attained is a problem requiring further study. The observation of Carey[3] that expansion and extension of the processes of the motor end plate of intercostal muscles of the rat occurs during hypercapnia suggests

* This work was supported by a grant from the Horace H. Rackham Foundation.

[1] Mason, A., and Gesell, R., *Fed. Proc.*, 1942, **1**, 58.

[2] Gesell, R., Mason, A., and Brassfield, C., *Am. J. Physiol.*, 1944, in press.

[3] Carey, E. J., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 67.

an additional role of acid as a nervous integrator. Enlargement of motor end plates in respiratory muscles might readily increase the amount of electrotonic current generated by the plates. A differential effect on the response of respiratory and non-respiratory muscles to the electrotonic current generated at the motor end plate is another possibility.

The differential effects of a common increase in cH may be of biological significance in preventing excessive use of locomotor muscles and assuring an adequate pulmonary ventilation for the needs of the body as a whole.

14634 P

Effect of Various Digitalis Glycosides upon the Cardioinhibitory Action of Acetylcholine.

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We have previously reported that in the dog 10% of the lethal dose of a certain tincture of digitalis abolished the cardiac inhibition produced by acetylcholine.[1] A preliminary investigation to determine whether this was due to some one or other of the known glycosides in digitalis purpurea has not been conclusive, but indicates that the glycosides do not behave identically in respect to their modification of the response to acetylcholine. To test this question further, the 3 glycosides of digitalis lanata, Lanatoside A, Lanatoside B, and Lanatoside C were employed.*

Anesthetized dogs were used. The minimal dose of acetylcholine bromide required to produce cardiac inhibition after injection into the femoral vein was determined. This varied in different animals from 0.1 to 1.0 mg. As the variation in an individual normal animal when tested at various intervals of time was occasionally as much as 50%, provision was made for such variation by using twice the minimal effective dose of acetylcholine. The cardio-inhibitory action of acetylcholine was determined from the carotid blood pressure tracing and by electrocardiograph tracings. An estimated 1/12th of the lethal dose of a glycoside was injected via the femoral vein, and similar doses were injected at 10-minute intervals until the animal died. Five minutes after each injection the test dose of acetylcholine (*i. e.* twice the original minimal cardio-inhibitory dose) was injected and observations made as to whether this did or did not cause cardiac inhibition. The results in 10 animals with each glycoside are shown in Table I.

Lanatoside C invariably abolished the cardiac inhibition produced by acetylcholine and did this when an average of 62% of the lethal dose was given. Lanatoside A produced such an effect in only 2 out of 10 experiments and Lanatoside B in but 5 out of 10 experiments. A statistical comparison of the frequency with which the various glycosides abolished the cardio-inhibitory action of acetylcholine shows that the probability that the differences between Lanatosides A and C could be due to chance alone is 1 in 2800; between B and C is 1 in 61; and between A and B is 1 in 6.

These results have a bearing upon the question of the similarity of cardiac effect produced by various cardiac glycosides. It is well known that these agents vary as to their absolute potency, absorbability from the gastrointestinal tract and duration of action. However, it is generally assumed that their cardiac actions are qualitatively identical. In the present experiments a progressively increasing cardiac action from initial to lethal effect was produced in each case.

1 Wells, J. A., Dragstedt, C. A., Rall, J. E., and Ruge, D. A., *Fed. Proc.*, 1943, **2**, 93.

* The glycosides used in the present study were furnished to us through the courtesy of Sandoz Chemical Works, Inc.

TABLE I.
Effect of Lanatosides A, B, and C on the Cardio-inhibition Produced by Acetylcholine in Anesthetized Dogs.

	Lanatoside A			Lanatoside B			Lanatoside C		
	I	II	III	I	II	III	I	II	III
	.25	(—)	57	.82	(—)	100	.14	84	84
	.36	(—)	67	.25	80	80	.24	83	75
	.27	(—)	77	1.17	(—)	90	.54	85	85
	.34	(—)	93	.10	(—)	100	.24	55	55
	.66	58	33	.13	(—)	67	.22	50	50
	.33	60	70	.21	(—)	76	.17	43	43
	.49	(—)	86	.22	63	54	.26	50	50
	.40	(—)	62	.25	58	58	.23	67	67
	.43	(—)	38	.22	67	55	.30	45	45
	.65	(—)	35	.29	84	38	.23	56	56
Avg	0.42		62	.37		73	.26	62	62

I Lethal dose in mg per kilo. II. % of lethal dose which abolished the cardio-inhibitory effect of acetylcholine. III. % of lethal dose which produced gross cardiac irregularities. (—) Cardio-inhibitory effect of acetylcholine not abolished at any time.

If the cardiac effects of the various glycosides are qualitatively identical there should be a similar sequence of evolution of the characteristic phenomena in each case and the phenomena produced by a certain per cent of the lethal dose of one glycoside should be produced by the same per cent of the lethal dose of another. The results indicate that such is not the case and therefore an actual qualitative dissimilarity in cardiac action appears to exist.

No explanation for this apparent qualitative dissimilarity between the glycosides is offered, and it is not clear why each glycoside did not prevent the cardio-inhibitory action of acetylcholine when evidences of idioventricular rhythms appeared. Electrocardiograms show that the dose of acetylcholine used produces auriculo-ventricular block. While digitalis principles also depress auriculo-ventricular conduction, we have seen no evidence of an enhanced response to acetylcholine in the digitalized animals.

14635

Activity of Penicillin Against *Hemophilus ducreyi in vitro.*

FRANCO MORTARA, ROSE R. FEINER, AND ESTHER LEVENKRON. (Introduced by G. L. Hobby.)

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The activity of penicillin on *Hemophilus ducreyi* was studied *in vitro* by the serial dilution method.

Both a crude filtrate of a culture of *Penicillium notatum* and a standardized penicillin* were tested against this organism. The test cultures were 5 strains of *Hemophilus ducreyi,* three JS, LB, and BF, recently isolated in our laboratory from patients with chancroid, and two, JB and S, stock cultures. All strains were morphologically typical of *H. ducreyi* and were pathogenic for rabbits

* This penicillin was obtained from Dr. G. L. Hobby of the Department of Medicine, College of Physicians and Surgeons, Columbia University. It was prepared in the laboratory of that Department under permit of the War Production Board and had been standardized against penicillin received from Dr. R. Coghill of the Northern Regional Research Laboratories, Peoria, Illinois.

TABLE I.
Activity of Crude Penicillin Filtrate Against *H. ducreyi in vitro* in Serum Broth.

	Dilutions					
	1:160	1:320	1:640	1:1280	1:2560	Control
Streptococcus hemolyticus C203Mv	—	—	—	—	—	++++
Staphylococcus aureus 209	—	—	—	++	+++	++++
Hemophilus ducreyi JB	—	—	++	++++	++++	++++
" " S	—	—	++	++++	++++	++++
" " JS	—	—	—	++	++++	++++
Escherichia coli	++++	++++	++++	++++	++++	++++

— No visible growth.
+ to ++++ Relative turbidity for each organism after 24 hours as compared with the respective control.

by intradermal inoculation.† In addition, strain JB proved at the time of the experiment to be virulent for man on experimental inoculation into the skin.‡

Method. Cultures of *H. ducreyi* were grown in defibrinated rabbit blood at 35° C for 24-48 hours and stored in the refrigerator. Transfers were made once a week. Good growth was obtained by inoculation of the stock cultures into 10% rabbit serum nutrient broth and subsequent daily passage in this medium. *Staphylococcus aureus,* strain 209, was used as the control test strain for determining the inhibitory titer of the penicillin preparations. *Streptococcus hemolyticus,* penicillin susceptible,[2, 3] and *Hemophilus pertussis, Hemophilus influenzae* and *Eschericha coli,* penicillin resistant,[2, 3] were included for purposes of comparison. All organisms were tested for sensitivity to penicillin in 10% rabbit serum broth, with the exception of *H. influenzæ* which was grown in Levinthal's medium. In addition, penicillin sensitivity was tested on 10% rabbit defibrinated blood agar plates.

Crude penicillin filtrate was diluted 1:40 by adding 0.2 cc to 7.8 cc of broth. Serial twofold dilutions were then made in serum broth up to 1:2560. Each tube containing 1.8 cc of the proper dilution of penicillin was inoculated with 0.2 cc of a 10^{-1} dilution of a 20-hour culture. Control tubes without penicillin were inoculated similarly. Standardized penicillin was diluted in broth or Levinthal's medium to give the desired number of Florey units per cc. Each tube, as well as controls without penicillin, was then inoculated with 0.2 cc of a 10^{-1} dilution of culture, the final volume being 2.0 cc. Standardized penicillin was also incorporated in rabbit blood agar with duplicate plates for each concentration of penicillin used. All plates, including controls without penicillin, were divided into sections and streaked with 20-hour undiluted cultures. Observations were made after 24 to 48 hours' incubation at 35°C. The last dilution showing a clear fluid or no visible growth at the end of the 24 hours was considered the endpoint in the titration.

Results. An inhibitory action of penicillin on the growth of *H. ducreyi* was demonstrated with both the crude and purified preparations. The results of the experiment are shown in Tables I, II, and III.

The titration endpoints with both crude filtrate and standardized penicillin were in agreement with several previous tests in which the same substances had been titrated against the same organisms. All strains of *H. ducreyi* were of approximately the same order of sensitivity to penicillin as the test strain of *Staphylococcus aureus.* The greater inhibitory action of penicillin against *Streptococcus hemolyticus* than against either the Ducrey bacillus or *Staphylococcus aureus* is indicative of the different sensitivity of vari-

† This confirms the report of Maximova[1] that intradermal injection of *H. ducreyi* in rabbits is followed within 24-48 hours by a pustular lesion.

‡ Personal communication of Dr. A. J. Pereyra, Comdr. (M.C.), U. S. N.

1 Maximova, A. A., *Ann. Dermat. et Syphil.,* 1936, **7**, 840.

2 Fleming, A., *J. Path. and Bact.,* 1932, **35**, 831.

3 Hobby, G. L., Meyer, K., and Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 277.

TABLE II.
Activity of Standardized Penicillin Against *H. ducreyi in vitro* in Serum Broth.

	Units per cc										
	2.0	1.0	0.5	0.25	0.13	0.06	0.03	0.016	0.008	Control
Streptococcus hemolyticus C203Mv	—	—	—	—	—	—	—	—	+	+
Staphylococcus aureus 209	—	—	—	+	+	+	+	+	+	+
" " *	—	—	—	—	—	+	+	+	+	+
Hemophilus ducreyi JB	—	—	—	—	+	+	+	+	+	+
" S	—	—	—	—	—	+	+	+	+	+
" JS	—	—	—	—	—	+	+	+	+	+
" LB	—	—	—	—	—	+	+	+	+	+
" BF	—	—	—	—	—	+	+	+	+	+
" *pertussis*†	—	—	+	+	+	+	+	+	+	+
" *influenzæ**	+	+	+	+	+	+	+	+	+	+
Escherichia coli	+	+	+	+	+	+	+	+	+	+

— No visible growth.
+ to ++++ Relative turbidity for each organism after 24 hours as compared with the respective control.
* Tested in Levinthal's medium.
† Tubes were inoculated with undiluted cultures—results were read after 28 hours.

TABLE III.
Activity of Standardized Penicillin Against *H. ducreyi in vitro* in Blood Agar.

	Units per cc					
	2.0	0.5	0.13	0.03	0.008	Control
Streptococcus hemolyticus C203Mv	—	—	—	—	+	+
Staphylococcus aureus 209	—	—	—	+	+	+
Hemophilus ducreyi JB	—	—	—	+	+	+
" S*	—	—	—	+	+	+
" BF*	—	—	—	+	+	+
" *influenzæ*	++	++	++	+	+	+
Escherichia coli	++	++	++	+	+	+

— No growth.
+ Growth.
* Read after 48 hours, no visible growth being present after 24 hours.

ous organisms to the action of this substance. Similar observations have been made by other workers.[3] It may be difficult to make an accurate comparison of sensitivity since the number of organisms in the inocula used in these tests differed appreciably for different organisms. One must consider that *H. ducreyi* is a rather slowly growing organism and 24-hour cultures would therefore be expected to contain fewer organisms than similar cultures of either *Staphylococcus aureus* or *Escherichia coli*. It has been pointed out, however, that the number of organisms in the inoculum is of little significance in this regard.[4, 5]

[4] Abraham, E. P., *et al.*, *Lancet*, 1941, **2**, 177.

[5] Fisher, A. M., *Bull. Johns Hopkins, Hosp.*, 1943, **73**, 343.

It is of interest to note that the sensitivity of *H. ducreyi* to penicillin *in vitro* would seem to differentiate it somewhat from other organisms of the same group, such as *H. influenzae* and *H. pertussis,* which are penicillin resistant.

Summary and Conclusions. The activity of penicillin on the growth of 5 strains of *H. ducreyi* was tested *in vitro* by the serial dilution method. *H. ducreyi* was found to be penicillin sensitive. This sensitivity was less than that of *Streptococcus hemolyticus* and paralleled that of the test strain of *Staphylococcus aureus.*

14636

Studies on the Action of Penicillin. II. Therapeutic Action of Penicillin on Experimental Meningococcal Infection in Mice.

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The effect of penicillin on several experimental infections in mice has been investigated[1-4] but even though it has been found to be highly satisfactory in the treatment of cerebrospinal fever, its application to experimental meningococcal infection in a laboratory animal has not as yet been reported. The present study undertook to fill a gap in our knowledge about this valuable chemotherapeutic agent.

Methods. Meningococci were grown for 6 hours on agar slants, suspended in Gelatin-Locke's solution to a turbidity known to approximate 1 billion meningococci per ml and then titrated in 4% mucin* by 10-fold dilution for intraperitoneal injection in 1 ml volumes.[5] Penicillin† in aqueous or saline solution was administered in the following volumes: Subcutaneous—.2 ml, intramuscular—.1 ml, intravenous—.2 ml.

Experiment I. Relation of the Time of Administration of Penicillin to Its Effect on Experimental Meningococcal Infection. The first experiments were made with a stock strain of meningococcus which had been carried for a number of months on artificial media with frequent mouse-passages. It possessed maximal virulence for mice by ordinary standards, *i.e.* fewer than 10 meningococci in 1 ml of 4% mucin regularly initiated a lethal infection.

Mice were infected with 100,000 MLD (10^{-4} of the standard suspension which at 10^{-9} killed the controls). At intervals of ½, 2, and 4 hours thereafter, groups of 4 mice were injected intravenously or subcutaneously with penicillin in a single dose of 100 Oxford units each.

As shown in Table I, the route of administration of penicillin made practically no difference in the results. Of the 8 treated ½ hour after inoculation, only one survived; whereas among those treated at 2 and 4 hours, 8 of 12 survived.

In a series of 13 mice infected with the next lower inoculum of meningococci (10,000 MLD) and treated at the same intervals, only one, which was treated at ½ hour, failed to survive.

* Granular mucin, Type 1701-W, supplied by the Wilson Laboratories, Chicago, Ill.

† The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for experimental investigations recommended by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

1 Hobby, G. L. Meyer, K., and Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 285.

2 McKee, C. M., and Rake, G., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 275.

3 McIntosh, J., and Selbie, F. R., *Lancet*, 1943, **2**, 224.

4 Hac, L. R., *J. Infect. Dis.*, 1944, **74**, 164.

5 Miller, C. P., and Castles, Ruth, *J. Inf. Dis.*, 1936, **58**, 263.

TABLE I.
Penicillin Treatment of Mice Infected with a Stock Strain of Meningococci.
Each dose of penicillin—100 Oxford units.

Inocula	Penicillin intravenous Time after inoculation ½ hr	2 hr	4 hr	Penicillin subcutaneous Time after inoculation ½ hr	2 hr	4 hr
100,000 MLD*	23	22	46	22	72	
	23	46	S	22	S	
	27	S	S	26	S	
	46	S	S	S	S	
10,000 MLD				23	S	S
				S	S	S
				S	S	S
				S	S	S

* Mice inoculated intraperitoneally with 1 ml of a 4% mucin suspension (100,000-1,000,000 meningococci) of which less than 10 sufficed to initiate a lethal infection in controls.
Numbers represent hour of death.
S = survival for 72 hours.

TABLE II.
Penicillin Treatment of Mice Infected with a Recently Isolated Strain of Meningococcus.

Dose	No. of injections Time after inoculation	1 ½ hr	1 3 hr	2 3 and 6 hr	3 1½, 4, 6 hr
	Mice Inoculated with 100,000 MLD.*				
50 Oxford units		22	22	26	25
		22	22	46	S
		22	22	46	S
		22	22	S	S
					S
100 " "		22	22	22	
		22	22	22	
		22	22	46	
		22	22	S	
				S	
	Mice Inoculated with 10,000 MLD				
50 " "		22	22	S	
		22	22	S	
		22	22	S	
		25	53	S	
				S	
100 " "		22	22		
		22	25		
		22	30		
		26	S		

These results showed that mice infected with very large doses of meningococci could be treated less successfully immediately after inoculation than a few hours later. These unexpected observations were repeatedly confirmed.

Experiment 2. Influence of Virulence of the Infecting Strain. When the foregoing experiment was repeated with a recently isolated strain the following differences were observed. None of the mice inoculated with 100,000 MLD and treated with 1 dose of 100 units of penicillin, survived and only one of those inoculated with 10,000 MLD.

Two injections of 50 units each sufficed to save all the mice inoculated with 10,000 MLD, but only one of 4 inoculated with 100,000 MLD. Two injections of 100 units saved 2 of 5 mice inoculated with 100,000 MLD and three injections of 50 units saved 4 of 5.

These results indicate that mice infected with a recently isolated strain did not respond to penicillin as readily as mice inoculated with a stock strain, even though the MLD of all strains used was less than 10 meningococci by

this method.[5] Whereas mice infected with the stock strain recovered after a single dose of penicillin, those inoculated with comparable numbers of recently isolated meningococci required repeated doses of penicillin.

Experiment III. To determine the effect of penicillin on the progress of the experimental infection, mice were inoculated intraperitoneally with 10,000 MLD of a recently isolated strain, as in the foregoing experiment, and one and 3 hours thereafter, groups of mice (designated first and second groups) were injected with penicillin intramuscularly. A group of infected, untreated mice served as controls. At intervals a pair of mice from each group was sacrificed and cultured by inoculating a drop of heart's blood and a loopful of peritoneal fluid onto a blood agar plate. The results of the blood cultures showed that meningococci did not invade the blood stream until about 3 hours after inoculation and disappeared immediately after the intramuscular administration of penicillin. The results of the cultures of peritoneal fluid combined from 2 experiments are plotted in Fig. 1 and show: (a) that the administration of penicillin at one hour (1st group) did not cause as rapid a reduction in numbers of meningococci in the peritoneal cavity as did its administration at 3 hours. (2nd group) (b) The penicillin appeared to have been active for 2-3 hours. (c) The peritoneal cavity was not sterilized. (d) The surviving meningococci in the first group multiplied rapidly after the effect of penicillin had worn off.

Experiment IV. As the foregoing experi-

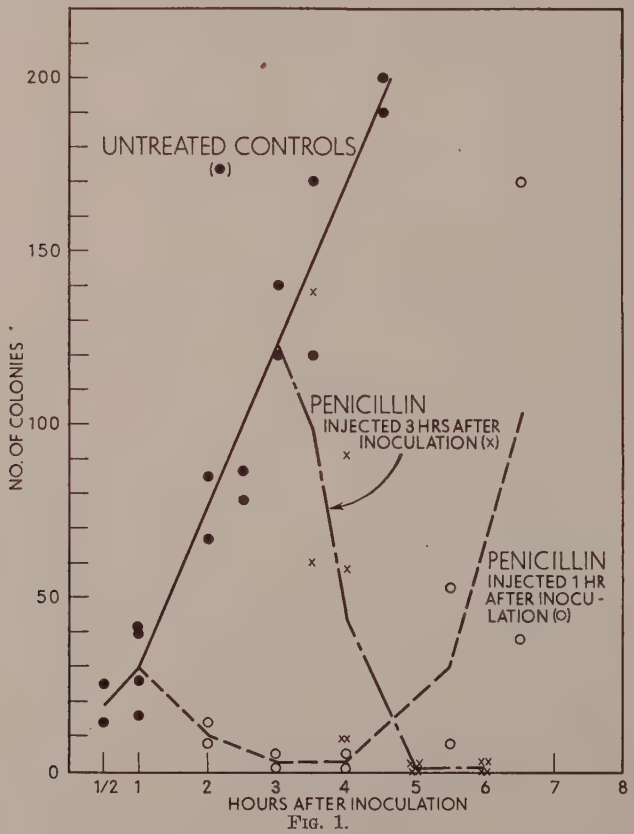

FIG. 1.

Effect of penicillin on progress of infection as indicated by the recovery of meningococci from the peritoneal fluid.

ment showed that the activity of penicillin lasted only 2 or 3 hours (see Fig. 1), the following experiment was designed to determine the time required for penicillin administered intramuscularly to reach the blood and peritoneal fluid in detectable amounts and the length of time it persisted.

Mice were injected intraperitoneally with 1 ml of sterile, 4% mucin and intramuscularly with 100 Oxford units of penicillin, and sacrificed at stated intervals thereafter. The heart's blood and peritoneal fluid obtained (.25 to 0.5 ml) were added to 1.5 ml of broth, titrated by 5-fold dilution and inoculated with 2×10^7 meningococci. The highest dilutions which inhibited growth showed that penicillin was detectable in the blood within 5 minutes (the first observation) and for about 2 hours thereafter. In the peritoneal fluid penicillin appeared within 5 minutes, rose for ½ hour to a somewhat higher level than in the blood and fell off about the end of the second hour. The penicillin determinations on the blood are subject to the limitations brought out in a later communication[6] which describes meningococcidal action of fresh serum.

Discussion and Summary. It should be remembered that the method of producing experimental meningococcal infection in the mouse involves the use of mucin, without which meningococci, even the most virulent strains, will not initiate a genuine infection. The method, nevertheless, lends itself to investigation of the action of penicillin *in vivo*. These studies have so far brought out the following points: Penicillin was equally effective if injected intravenously, subcutaneously, or intramuscularly. When given intramuscularly, it was detectable in the heart's blood within 5 minutes and persisted there for about 2 hours. Its diffusion into the peritoneal cavity occurred simultaneously and its concentration there rose to a relatively higher level than in the blood. (Cf. the clinical observations of Rammelkamp and Keefer[7] on its diffusion out of serous cavities). The penetration of penicillin into the peritoneal cavity may or may not have been affected by the presence of mucin.

The effect of penicillin on the bacterial population in the peritoneal fluid was an immediate decrease in numbers which was more striking if the infection had progressed for 3 hours than if the inoculum had been introduced only one hour before.

Infection with a stock strain of meningococcus, although fully virulent by ordinary standards, was more easily brought under control than infection with strains recently isolated from human cases of meningococcal infection. The latter required repeated doses of penicillin.

[6] Miller, C. P., and Foster, Alice Zimmerman, PROC. SOC. EXP. BIOL. AND MED., in press.

[7] Rammelkamp, C. H., and Keefer, C. S., *J. Clin. Invest.*, 1943, **22**, 425.

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Effect on Blood Agglutinins of a Polysaccharide Isolated from *Ascaris suum.*

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The inhibitory effect of a polysaccharide isolated from the pig roundworm, *Ascaris suum*, on the α and β agglutinins of human

* The authors wish to express their gratitude to Dr. Harry M. Rose of the Department of Medicine, College of Physicians and Surgeons, Columbia University, for valuable criticisms of the work. The authors are also indebted to Dr. A. S. Weiner, New York City, for supplying us with one of the human serums, and to Dr. C. LeF. Birch from the College of Medicine, University of Illinois, for the commercial guinea pig anti-Rh serum.

serums has been described.[1] The investigation described below has been carried out to determine the effect of the ascarid polysaccharide on anti-Rh, anti-M, anti-N, and on cold agglutinins.

Experimental Methods. Three serum samples with anti-Rh agglutinins were used, 2 of them obtained from 2 cases of erythroblastosis fœtalis, The third was a commercial preparation of guinea pig anti-Rh serum. Enough of the polysaccharide was added to these serums to bring its concentration to 4% and the mixture then incubated for 30 minutes in a water bath at 37°C.† One drop of a 2% suspension of known Rh positive and Rh negative cells respectively was added to 2 drops of each of the serums. Each serum was tested twice, one specimen with and the second without the polysaccharide. These mixtures were placed in Wassermann tubes and incubated in a water bath for one hour at 37°C, then centrifuged (500 revolutions per minute) for one minute and examined microscopically.

Analogous mixtures with and without polysaccharide were prepared also with commercial preparations of anti-M and anti-N serums.‡ One drop of a 2% suspension in saline of known M and N cells was added to 2 drops of each of the "treated" and untreated serums. The suspensions were left for 30 minutes at room temperature and examined microscopically at regular intervals.

In another experiment, a 4% concentration of polysaccharide was also added to 5 human serums with cold agglutinins. To 2 drops of the above mentioned "treated" and untreated serums, already placed in Wassermann tubes, one drop of a 2% suspension of the patient's own red cells was added the tube then stored in the icebox overnight at 6°C and examined microscopically on the following morning.

The serums from 5 different rabbits having cold agglutinins were also treated with the same amount of polysaccharide and similarly tested with A human erythrocytes and with the rabbit's own red cells. Previous to storage in the icebox, the untreated rabbit serums, plus the red cell suspensions, were incubated at 37°C for 30 minutes so as to exclude the possibility of the presence of α agglutinins. After exposure at 37°C for 30 minutes and at 6°C overnight, these suspensions were examined microscopically.

The addition of polysaccharide from *Ascaris suum* had no inhibitory action on the anti-Rh, anti-M, and anti-N agglutinins whenever these were present in the experiment just described. Agglutination of red cells by the homologous agglutinins was observed at about the same intensity in the serums with the polysaccharide as in the untreated ones (Table I).

The polysaccharide had no effect on the cold agglutinins present in either human or rabbit serums. Agglutination of all erythrocytes was seen in the "untreated" and "treated" human and rabbit serums after exposure to 6°C overnight. The rabbit serums showed no agglutinins against human A and rabbit cells after incubation at 37°C for 30 minutes (Table I).

Discussion and Summary. A 4% concentration of the ascarid polysaccharide added to human serums reduces the α and β agglutinins to a zero titer.[1] The results presented above indicated that the polysaccharide, when added to human serums at the same concentration at which it inhibits the α and β agglutinins, has no effect on anti-Rh, anti-M, anti-N, and on cold agglutinins. Thus, its action may be regarded as specific for α and β agglutinins. The fact that a polysaccharide with similar properties has been isolated from other parasites[2] suggests that certain infectious agents may have a closer relationship to human isoagglutinogens than is known at present.

The specific action of the ascarid polysaccharide may be used to advantage in the preparation of anti-Rh, anti-M, and anti-N serums that require the absorption of the α and β agglutinins. This is particularly true in human serums with anti-Rh agglutinins, where the α and β agglutinins have to be removed so that the serum could be used to detect Rh-positive or Rh-negative cells.

1 Oliver-González, J., *J. Inf. Dis.*, in press

† This and similar mixtures will be referred to as "treated" serums.

‡ Obtained from Lederle Laboratories, San Juan, Puerto Rico.

2 Oliver-González, J., and Torregrosa, Mercedes, *J. Inf. Dis.*, in press.

TABLE I.
Failure of a Polysaccharide from *Ascaris suum* to Inhibit Anti-Rh, Anti-M, Anti-N, and Cold Agglutinins.

Test materials				Positive agglutination with erythrocytes	
No. and source of serum samples	Agglutinins	Erythrocytes tested		Untreated serums	Serums treated with 4% polysaccharide
3 { 2 human, 1 guinea }	Anti-Rh	Group O Rh+	Group O Rh−	Rh+	Rh+
1 commercial	Anti-M	M and N		M	M
1 "	Anti-N	M and N		N	N
5 human	Cold agglutinins	Patient's cells		Patient's cells	Patient's cells
5 rabbit	Cold agglutinins, no agglutinins at 37°C	Group A and rabbit's own cells		Group A and rabbit's own cells	Group A and rabbit's own cells

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Poliomyelitis Virus in the Human Oro-pharynx.*

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In an effort to explain the transmission of poliomyelitis recent investigations have tended to emphasize the finding of virus in the feces. While successful attempts to demonstrate virus in the human nose and throat were made in earlier years these have virtually been abandoned in the face of readily isolated intestinal virus.[1] It is possible, however, that the technical difficulties of virus isolation have played an important role in emphasizing the apparent rarity of virus in the pharynx. The current communication deals with an attempt to vary the methods which have been employed in the past. Instead of the nasal or pharyngeal washing we have employed cotton swabs rubbed against the posterior wall of the oro-pharynx and the peritonsillar area. The swabs were then detached, dropped into a fluid-tight container with 1 cc of sterile water, and stored on dry ice. An average of 2 swabs was obtained from each subject.

The subjects were patients in the New Haven Hospital admitted during the late summer of 1943. Twenty different specimens were obtained at various times during the first week of the acute illness.‡ Patients were paralytic or non-paralytic. While it has been impossible to test the entire series of swabs, 14 specimens have been examined to date and it is felt that the results are of sufficient interest to merit a preliminary report at this time.

The technique of inoculation was as follows. It had previously been shown that elution from cotton or mouse cord emulsion containing Lansing virus was most complete at pH 8. On the other hand, mouse inoculation was more successful with the eluate ad-

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

† National Research Fellow.

‡ We wish to thank Dr. Robert Ward for valuable assistance in collecting some of this material.

1 Vignec, A. J., Paul, J. R., and Trask, J. D., *Yale J. Biol. and Med.*, 1938-39, **11**, 15.

TABLE I.
Poliomyelitis Virus in Throat Swabs.

Patient	Sex	Age	Type	Day of disease	Exp. result
Houde	♀	6	Non-paralytic	1	+
Johnson	♂	15	"	2	+
Broccoly	♀	11	"	2	+
Gronin	♂	10	"	1	—
Doer	♂	—	"	1	—
Gajewski	♀	12	"	2	—
Stowe	♂	11	Paralytic	2	+
Mari	♂	10	"	2	+
Stone	♀	6.5	"	2-3	—
Noa	♂	8	"	1	—
Shuman	♂	15	Bulbar	6	—
Morgan	♂	15	"	2	+
Lanette	♀	13	"	4-5	—
Hill	♂	13	Fatal Bulbar	1	+

justed at pH 6.[2] The throat swabs were accordingly eluted in phosphate buffer at pH 8, the fluid being pressed out of the cotton in a syringe. The eluate was then brought to pH 6 and treated with 20% ether in the ice box until sterile (usually 36 hours) at which time the ether was removed. In no case was more than 1.1 cc of inoculum obtained, which made it possible to give the entire specimen by intracerebral inoculation to a single monkey. Rhesus monkeys of 8-10 lb. were used. Under ether anaesthesia a trephine hole was made over the sagittal suture just posterior to the coronal sutures. It was thus possible through this single opening to place half of each inoculum into each lateral thalamus. This region was chosen because of its relatively high susceptibility and its deep position, which minimized regurgitation of the inoculum.

The 14 specimens tested were unselected from the larger series and are listed in Table I. Of this group, 7 (50%) produced typical poliomyelitis in the test rhesus monkeys. Six of the 7 animals were paralytic. Microscopic sections have shown characteristic lesions in each case and although virus studies have not been completed there seems little doubt regarding the nature of the infectious agent. The positive findings were equally distributed between paralytic and non-paralytic human cases.

Summary. Throat swabs were taken from 14 cases of human poliomyelitis during the first week of the disease. Seven of these (50%) were found to contain active virus.

[2] Wenner, H. A., unpublished.

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N. dibutyl Succinate as a Solvent for Parenteral Injection.

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Recently we have had occasion to test the toxicity of some water insoluble drugs. Lipschitz *et al.*[1] after extensive studies with various organic esters stated that *N. dibutyl* succinate showed a minimal toxicity when injected intramuscularly into rats and believed the agent suitable as a solvent for parenteral injections. This agent was selected for the solvent of our drug and was injected intra-

[1] Lipschitz, W. L., Upham, S. D., Hotchkiss, C. N., and Carlson, G. H., *J. Pharm. and Exp. Therap.*, 1942, **76**, 189.

peritoneally into rats.* Using constant amounts of the solvent with varying amounts of the drug to be tested, it was observed that all the rats behaved in a very peculiar manner, regardless of drug dosage. The solvent alone was tried by intraperitoneal injection and it was found that the solvent accounted for all the reactions observed.

A series of 20 albino rats weighing from 200 to 250 g were injected with the *N. dibutyl* succinate in graded amounts from 0.5 cc to 10 cc per kg intraperitoneally. The first effect observed was a tendency to gnaw the hind toes, then the rats assumed a somewhat flat crawling position, tending to drag the hind limbs. In a few minutes all the rats became sedated, and dropped off to sleep, with sudden rousing, dragging themselves about the cage and relapsing again into sleep. Respiration was quite markedly decreased. The minimal doses produced this effect lasting about one hour while the effects persisted for nearly 3 hours with the larger doses. All the animals recovered.

In view of the desirability of checking these observations with material from a different source, we obtained a second sample of the solvent from the Eastman Kodak Company. Twenty albino rats of about 200 to 250 g were selected and 10 were injected with the Eastman product and 10 with the original sample in doses of 0.5 cc to 5 cc per kg intraperitoneally. No consistent difference could be observed in the behavior of the rats with the different samples.

The toxicity of the *N. dibutyl* succinate was then extended to rabbits. Eight albino rabbits weighing from 2.8 to 3.4 kilos were injected intramuscularly with doses ranging from 0.33 cc to 2 cc per kg and 6 rabbits were injected intravenously in pairs with 0.5 cc, 0.75 cc and 1 cc per kg. No effects other than marked dilatation of the ear vessels could be observed in the rabbits injected intramuscularly. In the series of intravenous injection, dosages of 1 cc per kg caused the animals to give a loud cry in about 1½ minutes, had a slight convulsion and died of respiratory failure in a few minutes. The 0.75 cc per kg dosage produced slight convulsions in a few minutes, then considerable sedation and respiratory depression and death in 9 and 12 minutes respectively. The 0.5 cc per kg dose did not produce any convulsions, but the animals were sedated and respiration markedly depressed. They were returned to the stock cages after 2 hours in what appeared to be normal condition, but both were dead the next morning.

It is apparent that *N. dibutyl* succinate when rapidly absorbed is a toxic agent, and is not suitable as a parenteral solvent. When injected intramuscularly it shows little toxicity.

* The original sample of N dibutyl succinate was supplied by E. Bilhuber, Inc.

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A New Test (Blocking Test) for Rh Sensitization.*

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As was first shown by Wiener and Peters,[1] sensitization of Rh-negative individuals against the Rh factor can often be detected by *in vitro* tests for anti-Rh agglutinins in the individual's plasma. However, it was soon found[2,3] that there are many Rh-negative patients who are strongly sensitized to the

* Aided by a grant from the United Hospital Fund of N. Y. C.

1 Wiener, A. S., and Peters, H. R., *Ann. Int. Med.*, 1940, **13**, 2306.

2 Wiener, A. S., *Arch. Path.*, 1941, **32**, 227.

3 Levine, P., Burnham, L., Katzin, E. M., and Vogel, P., *Am. J. Obst. and Gyn.*, 1941, **42**, 925.

TABLE I.
Tests on a Series of Human Sera for Anti-Rh Blocking Antibodies.

Experiment	Test cells* (Group O)	Tests with sera from Rh-positive individuals							Tests with sera from Rh negative mothers of erythroblastotic babies					
		1	2	3	4	5	6	7	8	9	10	11	12	13
1	Rh_1	++±	++	++	++±	++±	++	+±	—	++	++	—	+±	—
2	Rh_2	++±	++	++	++	++	++	++	—	++	++	—	++	—

One drop of the serum being tested was mixed with a drop of test cells (2% suspension) in a small, narrow test-tube and the mixture allowed to interact in a water-bath at 38°C until sedimentation was complete (30 to 60 minutes). In experiment 1, the supernatant fluid was then removed, and a drop of diluted (1:5) anti-Rh_0 serum (original titer 60) was added to each tube. In experiment 2, the anti-Rh_0 serum was added directly without removing the supernatant. The tubes were shaken and then reincubated until sedimentation was complete, and the reactions were read grossly by inspecting the sediment in each tube, and microscopically after gentle shaking. Sera 8, 11, and 13 contain blocking antibodies.

* For nomenclature of the Rh blood types and Rh antisera, see Wiener, A. S., *Science*, 1944, **99**, 532.

Rh factor, as proved by the occurrence of an intragroup hemolytic transfusion reaction or a baby with erythroblastosis (hemolytic disease of the fetus and newborn), yet the plasma does not contain demonstrable anti-Rh agglutinins. The purpose of this paper is to describe a new *in vitro* test, the "blocking test," with the aid of which Rh sensitization can be detected in many of these problem cases.

The first blocking experiments were tried in 1941, when retests of some of the stored post-transfusion sera from the patients described by Wiener and Peters the year before showed these sera to be no longer active. It occurred to the writer that the antibodies might still be present and capable of combining with the test cells but incapable of agglutinating the cells. When the mixture of test cells and apparently inactive serum was first allowed to combine and subsequently active (capable of agglutinating Rh+ cells in control experiments), anti-Rh serum was added, it was found that the test cells were not agglutinated, apparently because the action of the active agglutinin had been blocked. However, the results obtained were irregular, and therefore experiments on this "blocking test" for Rh antibodies, which is a counterpart of the inhibition test for haptens and group-specific substances, were temporarily abandoned. Recently, when more satisfactory anti-Rh testing sera became available, the experiments were resumed. Tests have been carried out on a number of patients with erythroblastotic babies where the usual tests for anti-Rh agglutinins were unsuccessful, and in most of the cases clean-cut blocking reactions have been obtained, proving the presence of a special sort of anti-Rh isoantibody.

Table I presents the results of some blocking tests on a series of Rh-negative patients who have had erythroblastotic babies, as well as a control series of Rh-positive individuals. The technic of the test is simple: First, one drop of a 2% suspension of Rh-positive cells and a drop of the patient's serum are mixed in a small test tube and allowed to react in a water-bath at 38°C for 30 to 60 minutes. Then a drop of a suitable dilution of an active anti-Rh serum is added, and after an additional incubation period of 30 to 60 minutes, the reactions are read. If blocking antibodies are present, no agglutination will occur, or the clumping will be markedly weakened. That the reaction is specific follows from the fact that it has thus far been obtained only with sera from Rh-negative individuals sensitive to the Rh factor, such as mothers of erythroblastotic babies, and not with sera from Rh-positive patients or normal Rh-negative indi-

TABLE II.
Titration of Agglutinating and Blocking Anti-Rh Isoantibodies in the Serum of a Patient with an Erythroblastotic Infant.

Date of tests	Nature of tests	Test cells (Group O)	Dilution of patient's serum in test: Undil.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
6 days after delivery	Direct Titration	Rh_1	—	—	tr.	+	+±	+±	tr.	—	—	
		Rh_2	±	+±	++	++	++	++	++	+±	tr.	—
1 month after delivery	Direct Titration	Rh_1	—	—	—	tr.	+	—	—	—		
		Rh_2	—	—	—	—	—	—	—	—		
	Blocking	Rh_1	—	—	tr.	+±	++	++±	++±	++±		
		Rh_2	—	—	—	++	++±	++±	++±	++±		

The direct titrations were carried out in the usual manner, the readings being taken after 45 minutes in the water-bath at 38°C.

The blocking tests were carried out as described in Table I; in the tests with Rh_2 cells the supernatants were removed before adding the anti-Rh_0 serum, while in the tests with Rh_1 cells, the supernatants were not removed.

viduals not sensitized to the Rh factor. That the reaction is due to an antibody that becomes fixed to the test cells follows from experiment 1, Table I, in which the supernatant fluid was removed before the anti-Rh test-serum was added.

A number of human anti-Rh sera have been obtained which exhibit a marked prozone effect.[4,5] Taylor *et al.*[4] have attempted to explain the behavior of such sera on the basis of optimal proportions of antigen and antibody, but if this explanation were correct, it would be difficult to explain why the phenomenon does not occur more frequently. The present author's experiments described above suggest that such prozone phenomena may be due to the presence in such sera of a mixture of blocking and agglutinating antibodies, the latter being of higher titer. In support of this idea may be cited some observations recently made on a patient after she had given birth to an erythroblastotic baby (*cf.* Table II). Tests 6 days after delivery showed her serum to contain strong anti-Rh agglutinins exhibiting a distinct prozone effect. Direct tests performed only 3 weeks later were almost entirely negative for anti-Rh agglutinins, but quantitative blocking tests showed the presence of blocking antibodies of a titer 2 to 4. The most reasonable explanation of these findings is that immediately after delivery, this patient's serum contained a mixture of blocking and agglutinating antibodies, and that the latter diminished in titer more rapidly than the former. To test this hypothesis, the serum obtained one month after delivery was treated with Rh-positive cells in order to attempt to remove the blocking antibodies in case these masked the presence of low-titered anti-Rh agglutinins. It was indeed found that the absorbed serum then gave distinct and specific clumping, even though the original unabsorbed serum gave no or only faint reactions. Similarly, the serum obtained 6 days after delivery was improved instead of weakened by absorption with Rh-positive cells. Repetition of these absorption experiments gave irregular results, probably on account of the competition between the blocking and agglutinating antibodies.

If, as there is some reason to believe, of the two sorts of Rh antibodies, the blocking antibodies prove to be of greater clinical significance in the causation of erythroblastosis, this would serve to explain the puzzling lack of correlation between the titer of anti-Rh agglutinins in the maternal serum and the severity of the disease in the infant.

Recently, studies have been conducted on the titer and specificity of Rh blocking antibodies in various human sera. To date, the highest titer encountered was 64, and all the blocking antibodies had specificities corresponding to anti-Rh_0. Thus, type Rh_1 blood suspensions treated with such blocking sera gave reactions indistinguishable from type Rh′ blood with the three sorts of Rh

[4] Taylor, G. L., Race, R. R., Prior, A. M., and Ikin, E. W., *Brit. Med. J.*, 1942, **2**, 572.

[5] Levine, P., *Arch. Path.*, 1944, **37**, 83.

antisera, and in the same way type Rh_2 blood suspensions could be "converted" into type Rh'', etc.

The observations on blocking antibodies are of interest in connection with the problem of the nature of agglutination and precipitation reactions in general. According to Marrack's "framework" hypothesis,[6] the second stage as well as the first stage of these reactions is assumed to be specific, in contrast to the classic theory which postulates that the second stage is non-specific. Some observations on mixed agglutination reactions were previously reported which were more readily explained under Marrack's hypothesis than the classic theory.[7] The present observations can also be more easily explained under the framework hypothesis, if one postulates that the blocking antibodies are monovalent antibodies, in contrast to the usual agglutinating and precipitating antibodies which are assumed to be bivalent.[8]

In conclusion, it seems highly improbable that blocking antibodies are peculiar to the Rh factor. Doubtless, study of other antigen-antibody systems will reveal the existence of analogous phenomena.[9]

6 Marrack, J. R., Report No. 230, Medical Research Council, His Majesty's Stationery Office, London, 1934; second edition, 1938.

7 Wiener, A. S., and Herman, M., *J. Immunol.*, 1939, **36**, 255.

8 Pauling, L., Campbell, D. H., and Pressman, D., *Physiol. Rev.*, 1943, **23**, 203.

9 *Cf.* Jones, F. S., and Orcutt, M., *J. Immunol.*, 1934, **27**, 215; Kleckowski, A., *Brit. J. Exp. Path.*, 1941, **22**, 192; Hooker, S. B., and Boyd, W. C., *Ann. N. Y. Acad. Sci.*, 1942, **43**, 107.

14641 P

Effect of Picrotoxin on Electrical Excitability of the Respiratory Center.

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The inspiratory portion of the respiratory center has been accurately located in the ventral reticular formation of the medulla of the cat by means of the Horsley-Clarke stereotaxic instrument.[1] Electrical stimulation of this region results in a marked inspiratory response involving both thorax and diaphragm with fixation of the chest in inspiration. Since there is very little direct evidence as to the precise effect of respiratory stimulating drugs on the respiratory center itself, we have thought it worth while to study the influence of one of these drugs on the electrical excitability of this region.

Cats were anesthetized with sodium phenobarbital, 150 mg per kilo intraperitoneally. A tracheal cannula was inserted and the animal was allowed to breathe through a tube containing soda-lime into a small (400 cc) Krogh-type spirometer calibrated to 5 cc. The Horsley-Clarke stereotaxic instrument was placed on the head, and through a small burr hole in the skull a fine double pole electrode was inserted 12-13 mm posterior to the interaural plane and 1-2 mm lateral to the midline. By means of repeated stimulation as the electrode was lowered, the most sensitive region of the center was located.

The stimulus was applied to the center at the end of a normal expiration by means of a Goodwin stimulator[2] set to deliver a peak voltage of 1-5 volts at a frequency of 100 cycles per sec. Little spread of the current from the tips of the electrode appeared to exist since the response was markedly diminished by moving the electrode 0.5 mm up or

1 Pitts, R. F., Magoun, H. W., and Ranson, S. W., *Am. J. Physiol.*, 1939, **126**, 673.

2 Dusser de Barenne, J. G., Garol, H. W., and McCulloch, W. S., *J. Neurophysiol.*, 1941, **4**, 287.

down. The response of the center to a given stimulus was measured in terms of the inspiratory volume, and of course varied with different animals. It was found that a progressively increasing voltage produced an increasing response up to a maximum.

A submaximal response was chosen, usually 50-100 cc, and the voltage and frequency were kept constant for the duration of the experiment. Observations of the response of the center to this standard stimulus were made at intervals of 15 min. or less. Too frequent stimulation showed a tendency toward a decreasing response.

In 3 control cats, in which observations were made at 15 min. intervals for periods of 4-7 hours, it was found that there was a variation in the response (Fig. 1), but the maximum increase was 16%. In a series of 10 cats, after a control period of 1 hour, 0.25 mg of picrotoxin per kilo was injected intravenously. There occurred a somewhat delayed but abrupt and marked increase in the response of the center to the constant stimulus, followed by a very gradual decline (Fig. 1). In general, no change in the sensitivity occurred before 15 min. and in one instance, 23 min. was required. The response increased progressively and reached a maximum 30-135 min. from the time of injection of picrotoxin.

In 4 of the experiments, run for extended periods of time, it was found that the increased sensitivity persisted for from 6 to

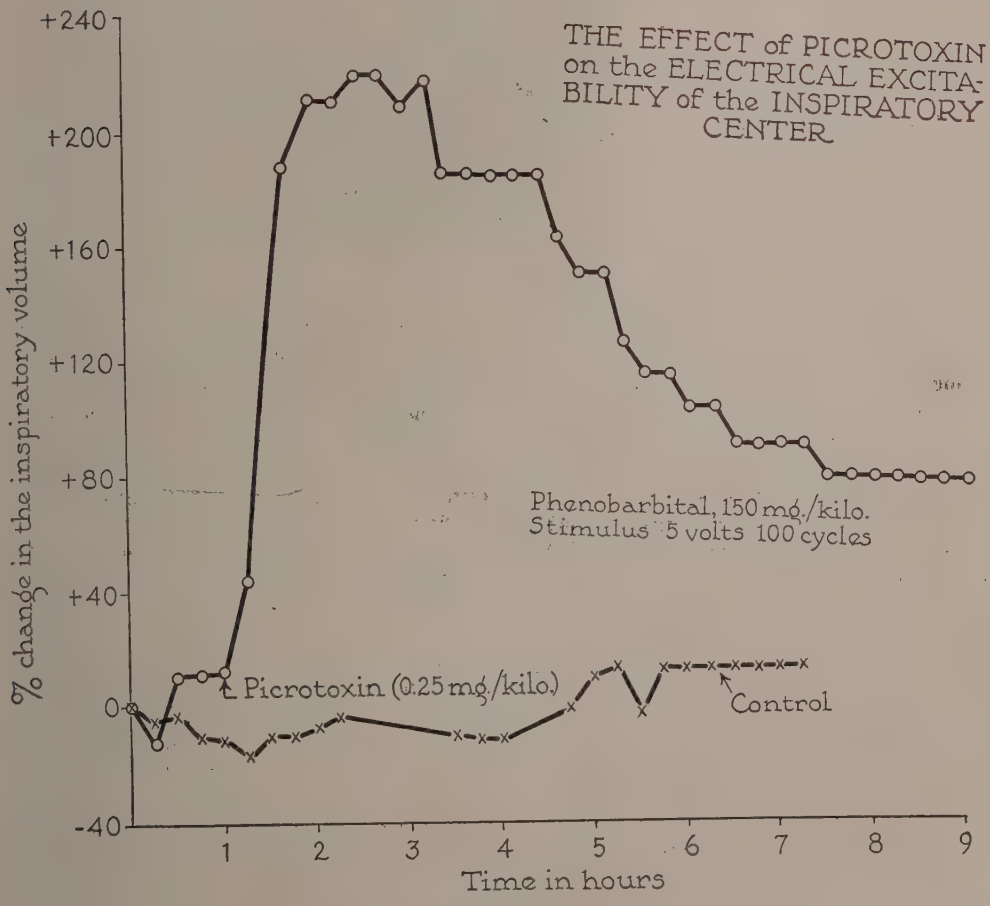

FIG. 1.

8 hours from the time of injection; at the end of this time the response became constant at a level either greater or less than the original. The maximum increase in the respiratory response varied from 30 to 222%. In certain instances the change in respiratory minute volume was also studied at frequent intervals and was found to correlate in a general way with the increased responsiveness of the center to electrical stimulation.

The delay in the onset of action of picrotoxin agrees with the observations of others and may well be due to a delayed passage of this substance into nervous tissue. However, the present prolonged response (average 7.5 hours) is not in accord with the usual beliefs regarding the duration of action of picrotoxin.[3, 4]

Summary. A technique has been described whereby electrical stimulation of the respiratory center can be employed to directly determine the influence on the respiratory center of various drugs affecting respiration. Picrotoxin has been shown to produce a marked and prolonged increase in the sensitivity of the inspiratory center of the cat to direct electrical stimulation. It is believed that the technique which has been described can be used to advantage to contribute additional information to our knowledge of drugs affecting respiration.

[3] Duff, D. M., and Dille, J. M., *J. Pharm. and Exp. Therap.*, 1939, **67**, 353.

[4] Das, S. C., *Quart. J. Exp. Physiol.*, 1939, **29**, 355.

14642

Bacteriostatic Action of Penicillin on Hemolytic Streptococci *in vitro*.*

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In previous communications[1,2,3] preliminary observations on the effect of penicillin on living cultures of hemolytic streptococci were reported. It was shown that the action of penicillin was either bactericidal or bacteriostatic depending on the experimental conditions. Under the influence of relatively large concentrations of penicillin, organisms when present in small numbers decreased in number at a constant rate until approximately 99% of the organisms originally present were destroyed. Penicillin was not inhibited by para-aminobenzoic acid, blood, serum, or peptone. Additional evidence suggested that penicillin was capable of destroying bacteria only when multiplication took place.

In the present communication evidence will be presented which extends these observations.

Experimental. Hemolytic streptococci (strain C203MV) were used throughout. In the first set of experiments the effect of varying amounts of penicillin was observed. A constant number of organisms was incubated in media containing amounts of penicillin varying from 0.0009 to 9 Oxford units per cc. The number of organisms per cc at intervals was determined by colony counts in pour plates. (Graph I).

With concentrations of 0.9 and 9.0 Oxford units of penicillin per cc the number of viable organisms remained unchanged for the first hour following which there was a steady decrease. With 0.09 units per cc there was an initial lag, followed by a slight increase and then a drop in the number of viable organisms.

* This work was presented in part before the New York and New Jersey Branches of the Society of American Bacteriologists, December, 1942.

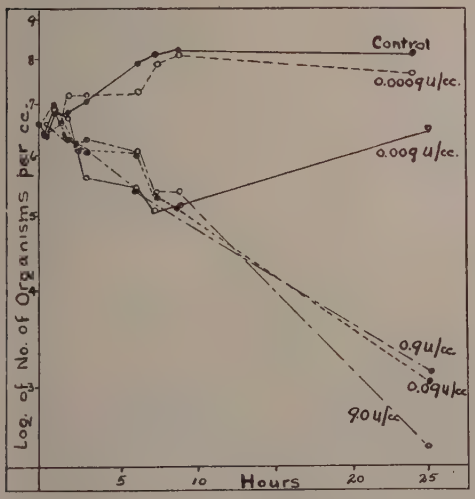
[1] Dawson, M. H., Hobby, G. L., Meyer, K., and Chaffee, E., *J. Clin. Invest.*, 1941, **20**, 434.

[2] Hobby, G. L., Meyer, K., and Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 277.

[3] Dawson, M. H., Hobby, G. L., Meyer, K., and Chaffee, E., *Ann. Int. Med.*, 1944, **19**, 707.

Graph I

Effect of Varying Concentrations of Penicillin on Hemolytic Streptococci in Vitro



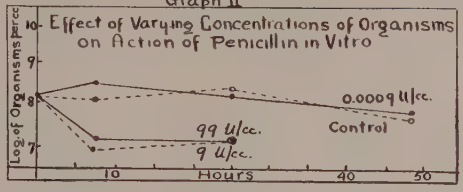
With 0.009 units per cc the results were similar for the first 9 hours of incubation but after 9 hours, multiplication again occurred. Concentrations of penicillin smaller than 0.009 units per cc caused only a slight slowing in the rate of growth.

In another series of experiments the effect of penicillin on varying concentrations of hemolytic streptococci was determined. Varying dilutions of an 18-hour culture were employed. The undiluted culture contained 200,000,000 organisms per cc, and the dilutions 2,000,000 and 40 organisms respectively. Penicillin was added to each dilution in amounts varying from 0.0009 to 0.9 Oxford units per cc and the cultures were incubated at 37°C. Controls without penicillin were incubated simultaneously. The number of organisms per cc at various intervals was determined as before by colony counts.

In plain broth with an initial concentration of 200,000,000 organisms per cc, there was no significant change in the number of viable organisms during the first 24 hours. After 48 hours the number of organisms diminished significantly. With 0.0009 to 0.09 Oxford units per cc similar results were obtained. In the presence of 0.9 to 99 units per cc the number of organisms decreased during the

Graph II

Effect of Varying Concentrations of Organisms on Action of Penicillin in Vitro



first 7 hours, then remained stationary. (Graph II.)

When the initial concentration of organisms per cc was lower—concentrations which permitted rapid multiplication in plain broth—no effect was observed with 0.0009 Oxford units per cc. With 0.009 units per cc a slowing of the rate of growth occurred and in the presence of larger amounts of penicillin direct killing took place. (Graphs III, IV.)

From the graphs it is apparent that the dilutions containing the greatest number of organisms showed killing as well as those containing fewer organisms—provided a sufficient amount of penicillin was used.

It is also apparent that the retardation of growth with small amounts of penicillin resembled that produced by sulfonamides in much greater concentrations. Penicillin in a concentration of 0.0009 Oxford units per cc produced an effect comparable with that of 100 micrograms of sulfadiazine per cc. In terms of pure penicillin[4,5] this represented an amount of 0.0005 μg of penicillin.

This bacteriostatic or bactericidal effect was apparent only with organisms sensitive to penicillin. A number of resistant strains of staphylococcus, pneumococcus, and *E. coli* were isolated. Resistant strains of staphylococci and *E. coli* were grown in broth for 18 hours at 37°C. The organisms were centrifuged, frozen, dried by the lyophile process, and ground with steel balls at a temperature of approximately −70°C. The resulting extracts were suspended in saline, again frozen and dried, and stored in dry ice. Solutions of these extracts were sterilized by Seitz filtration and tested for their effect upon penicillin. It was found that one microgram of freshly

[4] Coghill, Robt., *Chem. and Eng. News*, 1944, **22**, 588.

[5] Hunter, A. C., *J. Bact.*, 1944, **47**, 24.

Effect of Varying Concentrations of Organisms on Action of Penicillin in Vitro

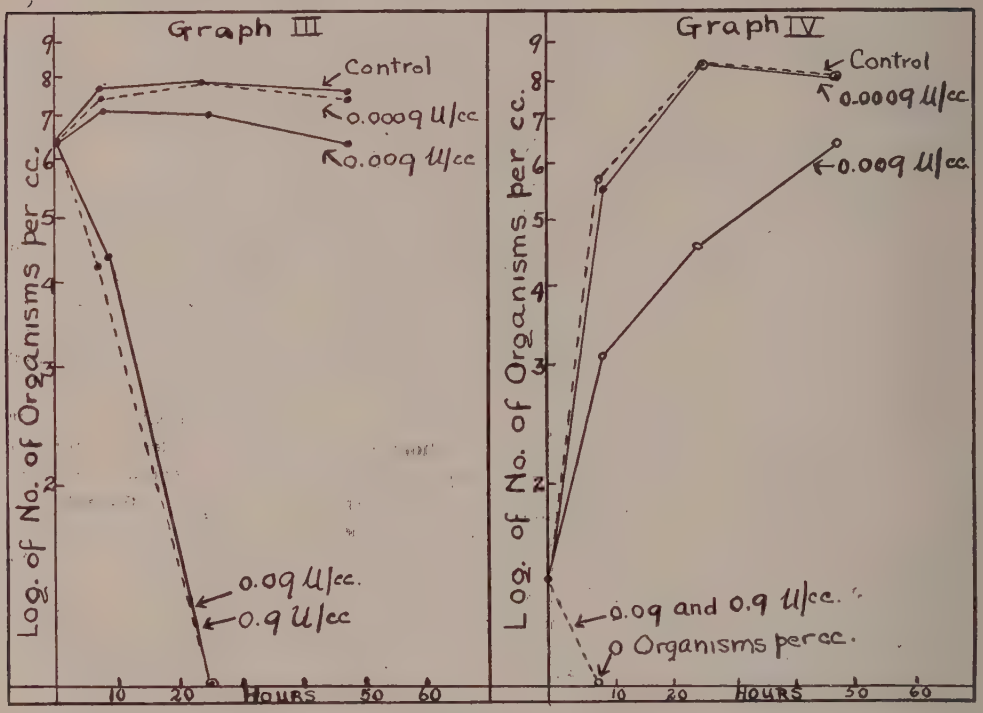


TABLE I.
Inhibition of Activity of Penicillin by Extracts of Bacteria.

Inhibitor			No. of organisms (× 1000) per cc			
Source	Amt (mg per cc)	Amt of penicillin (units per cc)	0 hr	2 hr	4½ hr	29 hr
—	—	—	2242	31000	31000	139000
—	—	2	2242	106	21	0
Pneumococcus	3.7	—	2242	19900	118500	221000
,,	3.7	2	2242	2550	46	0
E. coli	3.7	—	2242	13700	130000	297000
,, ,,	3.7	2	2242	15600	38000	130000

prepared crude extract from *E. coli* inhibited 0.01-0.1 unit of penicillin. The amount of inhibiting substance necessary to inactivate each unit of penicillin is therefore extremely small. (Table I.) Preliminary experiments showed that the inhibiting substance was partially or completely inactivated at 80°C for one hour, and that it was destroyed by M/200 sodium fluoride. A similar substance was isolated in 1941 by Abraham and Chain[6] who suggested that it was probably an enzyme. Although the number of strains tested was small, the inhibiting substance was found in

[6] Abraham, E. P., and Chain, E., *Nature*, 1940, **146**, 837.

all resistant strains, both Gram-positive and Gram-negative, and not in sensitive ones.

Summary. The effect of penicillin *in vitro* varies with the concentration of penicillin, the number of organisms present, and the rate of growth of the organisms. It is most effective when rapid multiplication takes place.

Penicillin is inhibited by a substance found in resistant strains of bacteria and not in the sensitive strains tested. The presence of this inhibiting substance in resistant strains and its absence in sensitive strains suggests that resistance to penicillin may depend upon the capacity of an organism to elaborate this substance. The degree of sensitivity of any strain not producing the inhibiting substance appears to be correlated with the rate of growth exhibited by that strain.

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Effect of Rate of Growth of Bacteria on Action of Penicillin.*

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In previous communications[1, 2] the effect of growth conditions of hemolytic streptococci on the action of penicillin were described. In the present report further observations of this nature will be reported.

Experimental. Hemolytic streptococcus (strain C203MV) was used throughout. Unless otherwise specified organisms were grown in a beef infusion phosphate buffered medium.

1. Conditions which enhance growth: Hemolytic streptococci were incubated in plain broth, in broth containing varying concentrations of defibrinated rabbits' blood, and in whole defibrinated rabbits' blood. A constant amount of penicillin was added to each of these. The number of organisms per cc was determined at various intervals.

In the presence of defibrinated rabbits' blood there was an increase in the rate of growth of hemolytic streptococci and a corresponding increase in the rate at which the organisms were destroyed by penicillin. The effect of 100% defibrinated rabbits' blood in contrast to plain broth is shown in Table I.

In a further series of experiments, the effect of serum, para-aminobenzoic acid, and dextrose was observed. Each of these substances also increased the rate of growth of hemolytic streptococci and caused a corresponding increase in the rate at which the organisms were destroyed by penicillin.

2. Conditions which inhibit growth: Hemolytic streptococci were incubated in media containing a constant amount of penicillin and varying concentrations of physiological saline. One volume of nutrient broth was diluted with one and with two volumes of physiological saline. A constant amount of penicillin and a constant number of organisms per cc were added. The number of organisms per cc was again determined at various intervals. Under these conditions there was a decrease in the rate of growth of hemolytic streptococci. There was likewise a decrease in the rate at which the organisms were destroyed by the penicillin. (Table II.)

Similar experiments were carried out in broth containing both penicillin and sulfadiazine. Sufadiazine was added to nutrient broth in amounts sufficient to give a concentration of 100 μg per cc. Penicillin was then added giving a concentration of 6.4 Oxford units per cc. Controls were run with nutrient

* This work was presented in part before the New York and New Jersey Branches of the Society of American Bacteriologists, December, 1942.

1 Hobby, G. L., and Dawson, M. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 178.

2 Hobby, G. L., Meyer, K., and Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 281.

TABLE I.
Effect of Blood on Rate of Action of Penicillin.

Medium	Conc. of penicillin (Oxford units/cc)	No. of organisms (× 1000) per cc			
		0 hr	2 hr	4½ hr	7 hr
Blood*	—	8200	69000	900000	880000
"	3.34	8200	200	165	84†
Plain broth	—	8200	19000	84000	60000
" "	3.34	8200	8000	1180	79†

* 100% defibrinated rabbit's blood.
† 0 per cc at 30 hours.

TABLE II.
Effect of Sodium Chloride on Rate of Action of Penicillin.

Amount of saline in broth %	Concentration of penicillin (Oxford units/cc)	No. of organisms (× 1000) per cc				
		0 hr	2¼ hr	4½ hr	7 hr	24 hr
—	—	6345	32000	152000	190000	215000
—	—	6345	1950	1400	12	0
50	—	6345	30500	127000	135000	185000
50	5	6345	3900	1740	230	0
66	—	6345	8800	54500	75300	245000
66	5	6345	3750	2700	690	200

broth alone, nutrient broth plus sulfadiazine, and nutrient broth plus penicillin alone. A 10^{-3} dilution of a 15 hour broth culture of hemolytic streptococci (strain C203MV) was then added, giving a final dilution of 10^{-4}. The number of organisms per cc was again determined at intervals.

During the first 5 to 7 hours of incubation, sulfadiazine alone had no effect on the rate of growth of hemolytic streptococci. During the same period penicillin alone destroyed about 90% of the organisms originally present. When incubation was continued further penicillin gradually destroyed the remaining viable organisms. During the first 5 to 7 hours, the combination of penicillin and sulfadiazine together killed at the same rate as penicillin alone. After 5 to 7 hours, the combination of penicillin and sulfadiazine destroyed the remaining organisms more slowly than did penicillin alone.

In another series of experiments hemolytic streptococci were incubated in the presence of sulfadiazine for 5 to 7 hours and then penicillin was added. Controls were prepared with nutrient broth alone, with nutrient broth containing sulfadiazine, and with nutrient broth to which penicillin was added after 5 to 7 hours' incubation.

The results again indicated that the presence of sulfadiazine caused a decrease in the rate of growth after 5 to 7 hours. Penicillin added to the sulfadiazine-containing medium during this phase of slow multiplication destroyed the organisms more slowly than when added to plain broth at the same stage in the growth cycle.

Further experiments were carried out under conditions which allowed little or no multiplication of the organisms. The effect of penicillin on varying concentrations of hemolytic streptococci, incubated at 4° C was determined. Varying dilutions of an 18-hour culture were employed. The undiluted culture contained 200,000,000 organisms per cc, and the dilutions 2,000,000 and 40 organisms per cc respectively. Penicillin was added to each dilution in amounts varying from 0.0009 to 0.9 Oxford units per cc and the cultures were incubated at 4° C. Controls without penicillin were incubated simultaneously. The number of organisms per cc at various intervals was determined by colony counts.

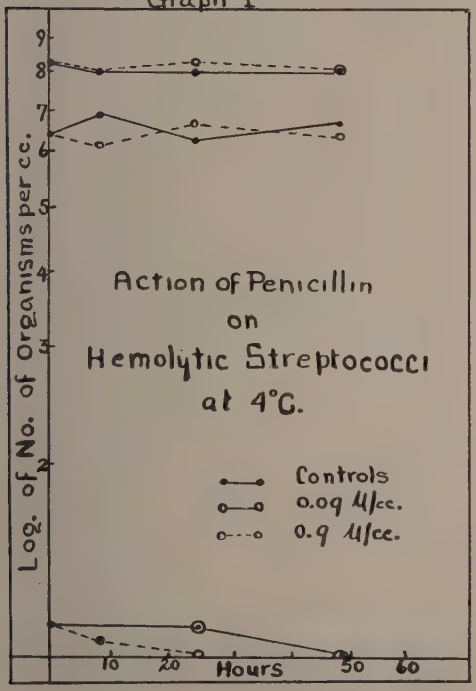
At 4° C no multiplication occurred regardless of the initial concentration of organisms. With an initial concentration of 200,000,000

TABLE III.
Effect of Medium on the Action of Penicillin.

Temp. of incub.	Time in hrs	No. of organisms (× 1000) per cc			
		Oxford units of penicillin per cc			
		0	0.1	10.0	100.0
		Unwashed Cells.			
37°C	0	92000	92000	92000	92000
	24	240000	145000	78000	64000
	48	19950	2750	805	1805
4°C	0	92000	92000	92000	92000
	24	190000	160000	245000	230000
	48	164000	103000	136000	130000
		Washed Cells.			
37°C	0	93000	93000	93000	93000
	24	505000	505000	1350	1350
	48	163000	69000	920	540
4°C	0	93000	93000	93000	93000
	24	170000	150000	175000	215000
	48	232000	121000	138000	140000

or 2,000,000 organisms per cc no killing occurred with amounts of penicillin up to 0.9 Oxford units per cc. With fewer organisms a gradual decrease in the number of organisms occurred with 0.9 units of penicillin per cc but not with 0.09 units per cc. (Graph I).

Graph I



In further experiments an 18-hour culture of hemolytic streptococci containing 93,000,000 organisms per cc was divided into two portions. One portion was centrifuged, the organisms washed 3 times with sterile broth and resuspended in an equal volume of broth. The other portion remained unwashed. Each was then subdivided into 4 portions. One of the 4 was kept as a control and varying concentrations of penicillin were added to each of the remaining 3. Incubation was carried out at 37° C and at 4° C.

The unwashed and washed cultures showed little multiplication in plain broth at 4° C; likewise they were unaffected by large amounts of penicillin at this temperature.

At 37° C little multiplication occurred in the unwashed cultures in plain broth; only a slight decrease in the number of organisms took place in the presence of relatively large amounts of penicillin. Washed organisms suspended in fresh broth also multiplied only to a slight extent. Under the influence of large amounts of penicillin the organisms were destroyed. However the destruction in washed suspensions proceeded more rapidly than that in the unwashed cultures. (Table III). It would seem that unwashed cultures contain some factor which inhibits the action of penicillin.

Observations showed that penicillin did not cause lysis of bacteria under conditions per-

mitting minimal multiplication. No absorption of penicillin onto the organisms could be demonstrated. Incubation of small numbers of organisms with penicillin under conditions suitable for active growth, however, resulted in the disappearance of the organisms. The failure to demonstrate these killed organisms by the usual staining technics suggested that they had undergone a subsequent lysis. This is in accord with the observations of other workers.[3, 4]

Summary. Conditions which increase the rate of growth of bacteria increase the rate at which penicillin acts. Conditions which decrease the rate of growth decrease also the rate at which penicillin acts. Penicillin is most effective when active multiplication takes place.

[3] Fleming, A., *Brit. J. Exp. Path.*, 1929, **10**, 226.
[4] Chain, E., *et al.*, *Lancet*, 1940, **2**, 226.

14644

Relationship of Penicillin to Sulfonamide Action.

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Ungar[1] reported on the synergistic action of both para-aminobenzoic acid and sulfapyridine on penicillin. In a previous communication from this laboratory,[2] it was stated that para-aminobenzoic acid increased the rate of action of penicillin whereas sulfadiazine decreased the rate of its action.

Experimental. Unless otherwise specified the Oxford strain of *Staphylococcus aureus* (strain H) grown in Knight's synthetic medium was used throughout the present study. Before use the culture was transferred 3 times in Knight's basic medium to which had been added M/100 nicotinic acid. A solution of penicillin was diluted serially in Knight s medium containing 100 μg of para-aminobenzoic acid per cc, in Knight's medium containing 20 μg of sulfadiazine per cc, and in Knight's medium containing 20 μg of sulfapyridine per cc, respectively. *Staphylococcus aureus* was inoculated into each tube in an amount sufficient to give a final dilution of 10^{-2} or approximately 10,000,000 organisms per cc. Incubation was carried out at 37°C for 24 hours. The last tube in which no visible growth occurred was accepted as the titer of the penicillin solution.

The titer of the penicillin in Knight's medium was 0.035 Oxford units per cc. With sulfapyridine the titer increased twofold to 0.018 units per cc. With para-aminobenzoic acid or sulfadiazine no increase was observed. (Table I.)

A similar experiment was carried out using hemolytic streptococci (strain C203MV) grown in beef infusion broth. No increase in the titer of the penicillin was observed with sulfadiazine, sulfapyridine, or para-aminobenzoic acid. (Table I.)

It was apparent that, although para-aminobenzoic acid and the sulfonamides may affect the rate of action of penicillin, they do not significantly affect the titer of penicillin.

Further preliminary experiments were carried out to determine any possible relationship between the action of penicillin and that of sulfadiazine or sulfapyridine.

West and Coburn[3] showed that sulfapyridine is capable of exerting a bacteriostatic

[1] Ungar, J., *Nature*, 1943, **152**, 245.
[2] Hobby, G. L., and Dawson, M. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 178.
[3] West, R., and Coburn, A. F., *J. Exp. Med.*, 1940, **72**, 91.

TABLE I.
Effect of Sulfonamides and Para-aminobenzoic Acid on Titer of Penicillin *in Vitro*.

Organism	Medium	Sulfapyridine, γ/cc	Sulfadiazine, γ/cc	Para-aminobenzoic acid, γ/cc	Titer of penicillin, Oxford units/cc
Staphylococcus aureus (strain H) 10^{-6}	Knight's basal medium containing M/100 nicotinic acid and penicillin	0	0	0	0.035
		20	0	0	0.018
		0	20	0	0.035
		0	0	100	0.035
Hemo. streptococcus (strain C203MV) 10^{-6}	Beef infusion broth containing penicillin	0	0	0	0.018
		20	0	0	0.035
		0	20	0	0.035
		0	0	100	0.035

TABLE II.
The Effect of Sulfonamides, Para-aminobenzoic Acid and Penicillin on Growth of *Staphylococcus aureus* in Synthetic Medium Containing Nicotinic Acid.

Organism	Knight's basal medium, cc	Nicotinic acid M/1000 cc	Sulfadiazine M/1000 cc	Sulfapyridine M/1000 cc	Para-aminobenzoic acid cc	Penicillin 10 Oxford units/cc cc	Growth 96 hr
Staphylococcus aureus (strain H), 10^{-2}	1.8	0	0	0	0	0	—
		.1	0	0	0	0	+
	1.8	.1	0	0.15	0	0	—
		.1	0.15	0	0	0	—
		.1	0	0	0.15 M/10,000	0	++++
	1.8	.1	0	0.15	0.15 M/50,000	0	+
	1.8	.1	0.15	0	0.15 M/10,000	0	+
Staphylococcus aureus (strain H), 10^{-2}	1.8	.1	0	0	0	.05	—
		.1	0.15	0	0	.05	—
		.1	0	0.15	0	.05	—
		.1	0.15	0	0.15 M/10,000	.05	—
		.1	0	0.15	0.15 M/50,000	.05	—

action in the presence of nicotinic acid but is inhibited by the presence of coenzymes. Experiments similar to those of West and Coburn were carried out using penicillin as well as sulfapyridine and sulfadiazine. As shown in Table II sulfapyridine and sulfadiazine were both active in the presence of nicotinic acid. Likewise penicillin was fully effective in the presence of nicotinic acid. Para-aminobenzoic acid greatly enhanced growth of the organisms in the basal medium containing nicotinic acid; it inhibited both sulfadiazine and sulfapyridine but did not inhibit penicillin activity.

Extracts of red blood cells were prepared according to the method of West and Coburn. As shown in Table III growth in Knight's basal medium was greatly enhanced by the addition of these extracts. Their presence inhibited the bacteriostatic action of sulfapyridine but did not inhibit the action of either sulfadiazine or penicillin.

It was apparent that penicillin, like sulfadiazine, was not affected by the presence of coenzymes.

Defibrinated rabbits' blood was shown to increase the rate of action of penicillin. There was no evidence, however, that extracts of red blood cells made from human blood produced any such effect. (Table IV.)

It has been shown by numerous investigators that organisms multiply at a normal rate for 5 to 7 hours in the presence of sulfadiazine

TABLE III.
Effect of Red Blood Cell Extracts on Action of Sulfapyridine, Sulfadiazine, and Penicillin on *Staphylococcus aureus in Vitro.*

Organism	Knight's basal medium cc	Red blood cell extract M/1000 cc	Sulfadiazine M/1000 cc	Sulfapyridine M/1000 cc	Penicillin 10 Oxford units/cc cc	Amt of growth 96 hr
Staphylococcus aureus (strain H), 10-2	1.8	0	0	0	0	—
		.15	0	0	0	++++
		.15	0	0.15	0	++++
		.15	0.15	0	0	—
		.15	0	0	0.05	—

TABLE IV.
Effect of Red Blood Cell Extract on Action of Penicillin *in Vitro.*

Temperature	Medium	Red blood cell extract cc	Penicillin 5.9 units/cc cc	No. organisms (× 1000) per cc 0 hr	No. organisms (× 1000) per cc 24 hr
37°C	Knight's basal medium 6 cc + nicotinic acid 0.1cc—M/100	0	0	228,950	264,000
		0.03	0	228,950	262,000
		0	0.07	228,950	257,000
		0.03	0.07	228,950	240,000
	,,	0	0	2,289	121,000
		0.03	0	2,289	255,000
		0	0.07	2,289	1.3
		0.03	0.07	2,289	1.2
4 °C	,,	0	0	228,950	490,000
		0.03	0	228,950	330,000
		0	0.07	228,950	280,000
		0.03	0.07	228,950	500,000
	,,	0	0	2,289	2,250
		0.03	0	2,289	1,575
		0	0.07	2,289	2,560
		0.03	0.07	2,289	1,445

or sulfapyridine and that bacteriostasis occurs only after this relatively long lag period. Repeated experiments have shown that penicillin is most effective during active multiplication but that the lag period before killing commences is short and the concentration of organisms unimportant so long as multiplication occurs and sufficient penicillin is present.[4]

In further experiments studies were carried out to determine the effect of concentration of organisms on the action of sulfadiazine and sulfapyridine.

Hemolytic streptococci were used throughout this experiment. Sulfadiazine and sulfapyridine were added to undiluted broth cultures containing 292,500,000 organisms per cc and to cultures diluted so as to contain 2,925,000 organisms and 29 organisms per cc respectively. The final concentration of each, sulfadiazine and sulfapyridine, was 99 μg per cc. Broth controls containing no drug were tested simultaneously. Incubation was carried out at 37°C.

In the presence of an initial concentration of 292,500,000 organisms per cc, a slight decrease in the number of organisms occurred in plain broth after 24 to 48 hours. The tubes containing sulfadiazine and sulfapyridine showed a similar change.

In the presence of an initial concentration of 2,925,000 organisms per cc, rapid multiplication took place in plain broth. Equally

[4] Hobby, G. L., and Dawson, M. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 178.

TABLE V.
Effect of Sulfonamides on Varying Concentrations of Hemolytic Streptococci (Strain C203MV) at 37°C.

Hr. of incubation	No. of organisms (× 1000) per cc		
	Sulfadiazine 99 μg/cc	Sulfapyridine 99 μg/cc	Broth
0	292,000	292,000	292,000
24	190,000	240,000	260,000
0	2,920	2,920	2,920
24	208,000	252,000	285,000
0	29	29	29
24	5,400	8,500	122,500

rapid multiplication occurred in the presence of either sulfadiazine or sulfapyridine.

In the presence of a small initial number of organisms per cc rapid multiplication occurred in plain broth whereas much slower multiplication occurred in the presence of sulfadiazine or sulfapyridine.

It is apparent that the sulfonamides were effective only after a lag period during which the organisms were in contact with the drug. On the addition of sulfadiazine or sulfapyridine after the organisms had passed into the phase of growth acceleration, no bacteriostasis took place. Neither sulfadiazine nor sulfapyridine acted on initially large numbers of organisms in spite of active multiplication. This is in contrast to the action of penicillin.

Summary. Under the experimental conditions used there was no evidence of a synergistic action between sulfadiazine or sulfapyridine and penicillin. Para-aminobenzoic acid did not increase the titer of penicillin although it does increase its rate of action.

14645

Inhibitory Effect of Certain Amino Acids on Growth of Young Male Rats.

STANLEY W. HIER, CLAIRE E. GRAHAM, AND DAVID KLEIN. (Introduced by E. M. K. Geiling.)

From The Wilson Laboratories, Chicago, Illinois.

Previous attempts to supplement gelatin with known amino acids so as to provide optimum growth have been unsuccessful (Jackson *et al.*[1] Kruse[2]). Threonine was not recognized at the time and we attempted to determine whether this was the necessary factor.

Arginine, histidine, lysine, valine, leucine, isoleucine, methionine, tryptophane, phenylalanine, and threonine were fed at levels in excess of Rose's minimum levels[3] with 20% gelatin (Group 2). A second group was fed the amino acids alone (Group 3). A third group was fed gelatin alone (Group 1).

The animals fed the supplemented gelatin made slight gains whereas loss of weight occurred in the same period on gelatin alone. The animals fed the amino acids alone grew better than those on the gelatin-amino acid diet, despite the fact that gelatin supplied "non-essential" amino acids. However, the rate of growth on the amino acids alone was not optimal.

It seemed to us that gelatin might be in-

[1] Jackson, R. W., Sommer, B. E., and Rose, W. C., *J. Biol. Chem.*, 1928, **80**, 167.

[2] Kruse, H. D., Day, H. G., and McCollum, E. V., *Am. J. Hygiene*, 1934, **19**, 260.

[3] Rose, W. C., *Physiol. Rev.*, 1938, **18**, 109.

TABLE I.
Nitrogen Supplement to Experimental Diets.

Group No.	Supplement g/100 g diet						
	Gelatin*	Amino acid mixture	Casein	Glycine (Merck)	*l*-Proline (Merck)	*dl*-Phenyl-alanine (Merck)	Fibrin
1	20						
2	20	†					
3		†					
4			20				
5			20	6			
6			20		5		
7			20			5	
8			50				
9			15				
10			15	6			
11							15
12				6			15

* Wilson's Pure-Food Gelatin.
† See Table II.
Diet 13—Stock diet.
Diet 14—94 g stock diet plus 6 g Glycine.

TABLE II.
Amino Acid Supplements to Diets 2 and 3.

Amino acid	Amt fed, g	Amt active, g	Rose essential amino acid requirements, g
dl-Methionine (Merck)	0.80	0.80*	0.6
l(+)Arginine HCl (Mearl)	0.40	0.33†	0.2
l(—)Histidine HCl (Paul Lewis)	0.60	0.49‡	0.4
l(+)Lysine HCl (Merck)	1.50	1.2 §	1.0
l(—)Tryptophane (Merck)	0.25	0.25	0.2
dl-Phenylalanine (Merck)	1.00	1.0 *	0.7
dl-Threonine (Merck)	1.50	0.75‖	0.6
dl-Valine (Merck)	1.80	0.90‖	0.7
dl-Leucine (Merck)	2.10	1.05‖	0.9
dl-Isoleucine (Merck)	1.20	0.60‖	0.5
Total	11.15	7.37	5.8
$NaHCO_3$	1.13 g ≅ 0.49 g HCl		

* Both isomers active.
† Contains 0.44 × .173 = 0.076 g HCl.
‡ " 0.60 × .190 = 0.114 g "
§ " 1.50 × .20 = 0.300 g "
‖ Only 50% active.

hibiting growth due to its marked amino acid imbalance, particularly its high content of glycine and proline. We therefore performed feeding experiments comparing growth on good basal diets with and without the additions of single amino acids.

Experimental Procedure. Male albino rats, 21 days old, were divided into groups of 2 of equal average weight, and fed the diets *ad libitum* assigned to the group. The diets, except for 13 and 14 contained the following basal ingredients.

Corn Oil	4 g
Salt Mixture	4 "
Celluration	4 "
Liver Conc. 1-20	1.5 "
Choline	0.3 "
Thiamine	0.3 mg
Pyridoxine	0.3 "
Riboflavin	0.3 "
Niacin	2.5 "
d-Ca Pantothenate	1.0 "

In addition, each rat received 2 drops of haliver oil per week.

The nitrogen supplements shown in Table I and the above basal ingredients were mixed with sufficient sucrose to make 100 g.

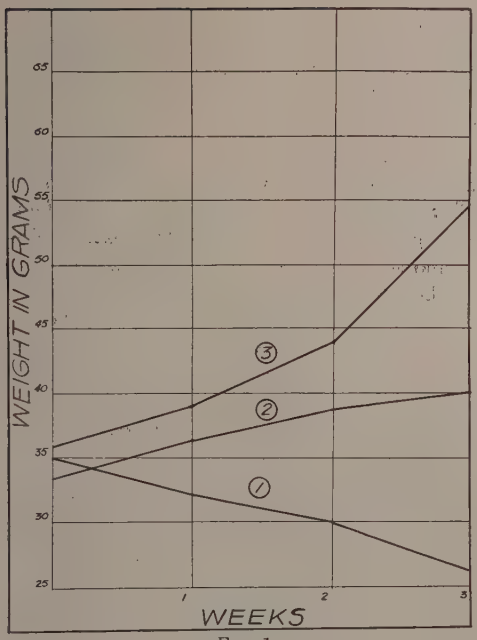

FIG. 1.

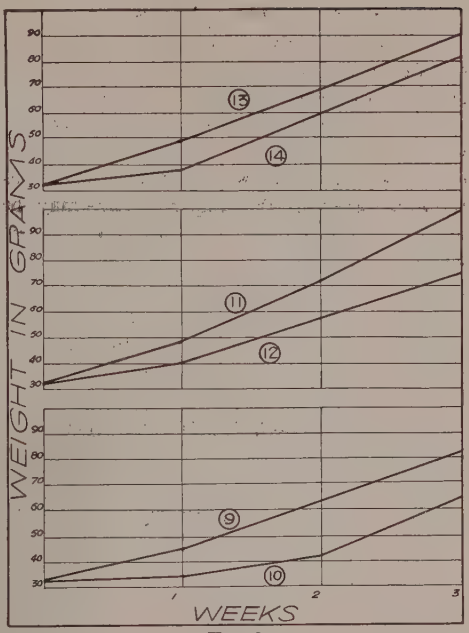

FIG. 3.

Results. Fig. 1 shows the superior growth obtained on amino acids (3) as source of nitrogen compared to growth on gelatin plus amino acids (2), or gelatin alone (1).

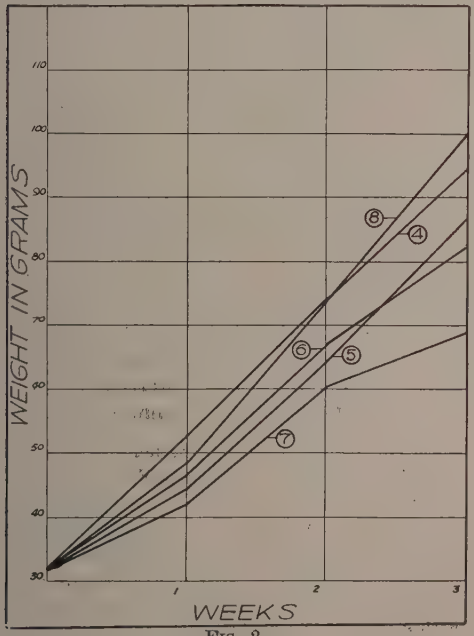

FIG. 2.

Fig. 2 shows the inhibitory effect of glycine on 20% casein diet (5) compared to 20% casein (4), and the inhibiting effect of 1-proline (6) and dl-phenylalanine (7) and non-inhibiting effect of 50% casein (8).

Fig. 3 shows the inhibitory effect of glycine on a 15% casein diet (10) compared to 15% casein alone (9), the inhibiting effect of glycine on 15% fibrin diet (12) compared to 15% fibrin alone (11) and the inhibiting effect of glycine on stock diet (14) compared to stock diet alone (13).

Discussion. The data indicate that glycine at 6% or 1-proline at 5% of the diets used have an inhibitory effect on growth and may explain the difficulty in supplementing gelatin with amino acids so as to make it adequate for growth.

dl-Phenylalanine at 5% has an inhibitory effect on growth even more severe than glycine and 1-proline.

These effects are not due to high nitrogen intake alone since 50% casein has no deleterious effect although it does supply considerably more nitrogen.

Sullivan *et al.*[4] reported growth inhibition and toxicity of glycine on a diet containing 4% casein at levels of amino acid from 2.5 to 20%. Jackson *et al.*[1] reported growth inhibition by 9% glycine as well as 35% gelatin when added to a normal casein diet. Their effects with glycine were not as marked as those reported in this paper. This may be due to their use of older animals (60-90 g) while our animals were started from weaning (35 g).

These data indicate the danger of imbalancing amino acid proportions by introducing high levels of a single amino acid even though it may be an essential amino acid. This is of particular importance in preparing experimental diets as some investigators have used high levels of glycine to balance nitrogen intake in protein and amino acid studies. Studies on other amino acids, to be reported, indicate that not all amino acids show this inhibitory effect.

Conclusion. 1. Gelatin will not allow optimum growth even when supplemented with all reported essential amino acids. In fact, less growth is obtained with gelatin plus amino acids than with the amino acids alone at the same level in the diet. 2. The presence of 6% glycine or 5% dl-phenylalanine or 5% l-proline in certain diets has an inhibitory effect on growth. 3. The inhibitory effect of glycine and proline on growth may explain the difficulty in supplementing gelatin for optimum growth.

[4] Sullivan, M. X., Hess, W. C., and Sebrell, W. H., *Pub. Health Rep.*, 1932, **47**, 75.

We wish to acknowledge and thank Miss Joan Staats for technical assistance.

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Failure to Demonstrate an Interrelationship Between Inositol and p-Aminobenzoic Acid in the Rat.

B. H. ERSHOFF. (Introduced by H. J. Deuel, Jr.)

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Martin[1] has pointed out that although purified rations containing 6 B complex factors (thiamine hydrochloride, riboflavin, pyridoxine, calcium pantothenate, nicotinic acid and choline chloride) maintained apparently normal nutrition in the Rockland strain rat, addition of p-aminobenzoic acid or inositol to the above ration at levels of 0.01 and 0.02% respectively precipitated syndromes that failed to develop if all 8 factors were present. In the present experiment rats of another strain were placed at weaning on a purified ration containing the 6 B complex factors listed above to which p-aminobenzoic acid and inositol were added in doses ranging from 0.05 to 1.0% of the ration, and the effect of feeding these diets determined.

Procedure and Results. The basal ration consisted of sucrose 73.5, Vitamin Test Casein* 22, and Sure's Salt Mixture No. 1[2] 4.5. To each kg of the above were added 20 mg thiamine hydrochloride, 20 mg riboflavin, 20 mg pyridoxine hydrochloride, 100 mg calcium pantothenate, 100 mg nicotinic acid, 1200 mg choline chloride and 5 mg 2-methyl-naphthaquinone. Daily supplements of 800 mg corn oil (Mazola), 0.5 mg alpha-tocopherol, and an A-D concentrate† equivalent to 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D were also administered.‡ Inositol and p-aminobenzoic acid

[1] Martin, Gustav J., *Am. J. Physiol.*, 1942, **136**, 124.

[2] Sure, Barnett, *J. Nutrition*, 1941, **22**, 499.

* S.M.A. Corporation, Chagrin Falls, Ohio.

† Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units vitamin D per gram.

‡ The vitamin content of the above ration is considerably in excess of minimum levels compatible with growth and reproduction.

TABLE I.
Composition of Experimental Diets and Number of Animals Employed.

Ration No.	Dietary supplement: Inositol %	Dietary supplement: *p*-Aminobenzoic acid %	Dietary base: Basal ration	Dietary base: Natural food ration*	No. of animals in group
A			X		6
B	.05		X		4
C	.5		X		4
D	1.0		X		6
E		.05	X		4
F		.5	X		4
G		1.0	X		6
H	1.0	1.0	X		5
I	1.25			X	4
J		1.25		X	4
K				X	6

* Purina dog chow supplemented once weekly with lettuce.

were incorporated in the above ration replacing an equal quantity of sucrose. The diets in Table I were employed.

Fifty-three male and female rats of the U.S.C. strain§ were placed at weaning (21 to 23 days) on the above rations. Animals were kept in metal cages with screen bottoms to prevent access to feces, and sufficient food was administered to assure *ad lib.* feeding. Feeding was continued for 8 weeks.

No significant difference in rate of growth or gross appearance was observed in any of the rats regardless of the dietary employed. At autopsy males in all dietary groups averaged 235±7 grams and females 190±10 grams, growth being slightly greater in the synthetic than the natural food series. Incorporation of inositol or p-aminobenzoic acid in doses ranging from 0.05 to 1.0% of the diet failed to exert any grossly visible deleterious effect. These findings are in marked contrast to those reported by Martin[1] who suggested that the incorporation of inositol in diets free of both this factor and p-aminobenzoic acid "stimulates the growth of organisms which utilize and destroy some member of the B complex, known or unknown, thus precipitating a deficiency of that factor. P-aminobenzoic acid either through stimulation or inhibition of bacterial growth precipitates an inositol deficiency." The results of the present experiment indicate that the mere presence of inositol or p-aminobenzoic acid in a purified ration containing the B complex factors listed above is not sufficient to assure a deficient state regardless of an apparent excess of these factors in the diet.

The present experiment differed from that of Martin in the strain of rat employed, the source of dietary fat, and the level and source of several vitamin supplements. The near marginal levels of thiamine, pyridoxine and choline employed by Martin as well as the factors enumerated above may be involved in explaining the diverse results.

Summary. No adverse effects were noted from the incorporation of inositol or p-aminobenzoic acid in a purified ration containing 6 B complex factors (thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, nicotinic acid and choline chloride).

§ A modified Wistar strain. From the animal colony, Department of Biochemistry, University of Southern California. Kindly provided by Dr. H. J. Deuel, Jr.

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Oxygen Consumption of Degenerated Optic Nerves.

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Michail and Benetato[1] found a 50 to 70% higher oxygen consumption in degenerated human optic nerves (from glaucomatous and atrophied eyes) than in optic nerves removed shortly after traumatization of the eye. Van Harreveld and Tyler[3] compared the metabolism of normal optic nerves of cats with that of eye nerves severed directly behind the eye 3 to 14 days before. A lower oxygen consumption of the operated nerves was found constantly. As the nerves examined by Michail and Benetato had probably been subject to degeneration much longer than the 2 weeks allowed for degeneration by van Harreveld and Tyler, the metabolism of optic nerves of cats, 6 months after transection was compared with that of the heterolateral normal eye nerves.

Methods. The optic nerve on one side was severed with aseptic precautions directly behind the eyeball. Six months later the oxygen consumption of the normal and degenerated nerves was determined in Warburg respirometers at 38°. The nerves were not minced but were left intact since previous experiments[3] had shown that the highest metabolism is found in undamaged nerves. The dura was removed with as little injury to the nerve as possible. The medium used was a glucose-Ringer, buffered with phosphates to a pH of 7.4. After the metabolism determination one part of each nerve was fixed and stained in osmic acid. The other part was fixed in Zenker's solution and later stained with hematoxyline eosin.

TABLE I.

Metabolism of Optic Nerves in Cubic Millimeters of O_2/Gram Wet Tissue/Hour.

No. of cat	Degenerated nerve	Normal nerve
1	460	590
2	530	480
3	430	510
4	510	470
5	340	510
6	320	470

Results. The results of the metabolism determination are shown in Table I. These figures give the oxygen consumption in mm^3 per gram of wet tissue during the hour between the 50th and 110th minute after the removal of the nerve from the animal. In 2 instances the degenerated nerve had a slightly higher, in 4 a lower metabolism than the normal nerve. The mean of the metabolism for the degenerated nerves was $430 \pm 20mm^3$, for the normal nerve $510 \pm 10mm^3$.

The degenerated nerves looked thinner and weighed less than the heterolateral normal optic nerves.

The osmic acid preparations of the normal side showed the fine nerve fibers with a thin myeline sheath typical for the optic nerve. In the degenerated nerve a few intact centrifugal fibers were found; the myeline of the degenerated fibers had, but for an occasional drop, disappeared. Few nuclei were found in the hematoxyline eosin preparations of the normal nerve. In the degenerated nerves there was, in all cases, a fair increase in the number of nuclei which were of two types, small well-stained nuclei (probably microglia), and larger, pale ones.

Discussion. An increase in metabolism in degenerated nerves would be conceivable on the basis of a relative (due to the resorption of axon material and myeline) and absolute increase of glia and connective tissue, assuming that these cellular elements have a

1 Michail, D., and Benetato, G., *C. R. Soc. Biol.*, Paris, 1936, **121**, 267.

2 Tobias, J. M., Clark, D. B., and Gerard, R. W., *Fed. Proceedings*, March, 1942, **1**, 85.

3 Van Harreveld, A., and Tyler, D. B., *Am. J. Physiol.*, 1942, **138**, 140.

higher metabolism than the axis cylinders, as is indicated for the glia by the work of Tobias, Clark and Gerard.[2] The above determinations do not bear out this expectation; the metabolism of the optic nerve in the cat, 3, 7 and 14 days[3] and 6 months after the beginning of degeneration is, if anything, lower than that of the normal nerve.

The degeneration in the optic nerves studied by Michail and Benetato was caused by pathological processes, and this may account for the higher metabolism found in their degenerated nerves. However, they minced their nerves, a process which reduces the oxygen uptake to a fraction of that of the undamaged nerve.[3] It is possible that mincing depresses the oxygen consumption of the normal nerve more than that of the degenerated one, which would lead to the erroneous conclusion that the degenerated optic nerve has a higher metabolism than the normal one.

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Site of Action of Indole in the Central Nervous System.

I. M. FEINBERG AND W. S. MCCULLOCH. (Introduced by Amedeo S. Marrazzi.)

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Despite the amount of work that has been done since Herter[1] first studied convulsant properties of indole, several points seemed worth clarifying. To this end, experiments were undertaken with the surgical assistance of Dr. Edward Davis.

In the first place, indole directly applied to the cortex of dog or cat produces a localized discharge resembling in many ways that seen following local strychninization. This is apparent from Fig. 1. As with strychnine, the concentration required for local application to elicit these results is great.

On the other hand, rapid intravenous injections of 20 mg per kg into dogs, cats and rabbits bring on convulsions which differ from fits induced by cortical excitation in that the motions of various muscles are not synchronized, and in that the axial and axio-appendicular, not the appendicular, muscles are most involved. Yanai[2] has reported that seizures occur after decerebration, to which we can add that they are not significantly altered by decerebration.

After transection of the spinal cord, the seizures may be more pronounced and begin at lower concentrations of indole either below or above the transection. Frequently, following transection of the neuraxis at any level, both portions are involved independently. Fig. 2 exemplifies this in the decerebrate preparation. The top record is from the cortex, and the bottom is from the hind limbs. They show cortical electrical discharges unrelated to the twitches of the hind limbs.

Under full ether anaesthesia, several dogs and cats were prepared by inserting electrodes to follow the electrical activity and the oxygen tension of the cortex and by installing tracheal and arterial cannulae. Several hours were allowed for recovery from ether. They were then paralyzed with dihydro-β-erythroidine hydrobromide and given artificial respiration. Thereafter, 18 to 30 mg of indole per kg, dissolved in olive oil or in less than 1 cc of propylene glycol, was given intravenously. The ensuing alterations in circulation produced corresponding changes in the O_2 tension of the cerebral cortex. On a few occasions, a few sharp diphasic spikes appeared in the record of the cortical activity, but at no time was a typical cortical seizure recorded, nor any decreases in O_2 tension ex-

1 Herter, C. A., *N. Y. Med. J.*, 1898, **68**, 89.

2 Yanai, Binici, *Tohoka J. Exp. Med.*, 1935. **25**, 401.

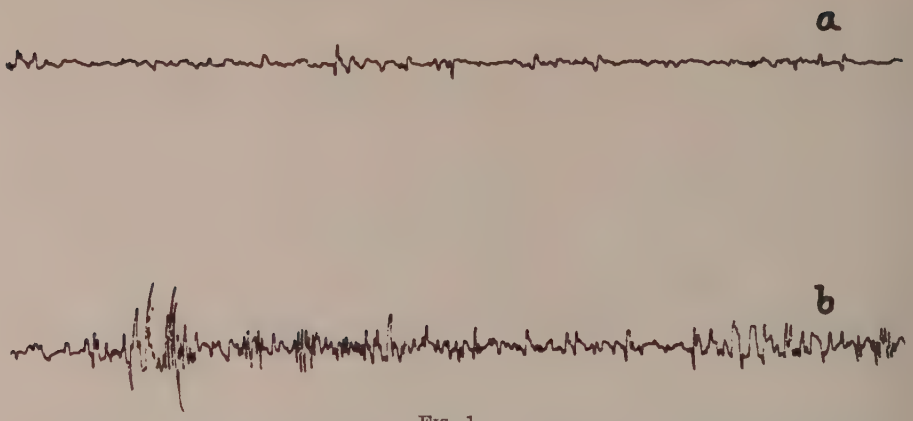

FIG. 1.

Cut No. 5. April 29, 1943. Cortex exposed and tracheal cannula installed under ether anesthesia. Four hours later—artificial respiration established under dihydro-β-erythroidine-hydrobromide. E.E.G. record (a) prior to local application of indole, (b) 7 minutes after local application of indole.

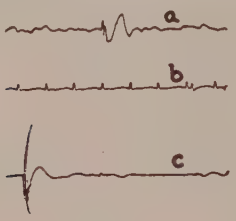

FIG. 2.

Dog. August 20, 1943. Under nembutal. Decerebration at level of superior colliculus. Synchronous records after intravenous injection of 25 mg of indole per kg. Whole dose in 2.5 cc propylene glycol. (a) E.E.G. from right hemisphere, (b) E.K.G. from both forelimbs, (c) E.M.G. from both hindlimbs.

cept those referable to the changes in circulation.[3-6]

With the larger doses of indole, the electrical record exhibited a late decline in both amplitude and frequency accompanied by a high O_2 tension.

Since indole has been shown to exert its convulsant action of the nervous system,[7] the experiments reported can be taken to indicate (1) that indole can act on many portions of the central nervous system, (2) that the thresholds of the various portions are all low and sufficiently nearly equal for other, uncontrolled, variables to determine which is the lowest, (3) that the characteristic motor seizure is certainly of subcortical and may, and in certain cases must, be of spinal origin.

[3] Ott, Isaac, and Ulman, Joseph F., *Therap. Gaz.*, 1907, **23**, 20.

[4] Danilewsky, B., *Pflüger's Arch.*, 1908, **125**, 361.

[5] Ets, Harold N., and Feinberg, I. M., *Proc. Pharm. Exp. Therap.*, 1939, 11.

[6] Ets, Harold N., and Feinberg, I. M., *Am. J. Physiol.*, 1942, **136**, 647.

[7] Yanai, Binici, *Tohoka J. Exp. Med.*, 1935, **25**, 385.

14649

Behavior in Amblystoma Larvae Lacking Forebrain, Eyes and Nasal Placodes.

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Observations have been made upon the feeding behavior in Amblystoma larvae devoid of the forebrain, eyes and nasal organs. The anlagen were removed from 60 embryos in Harrison's stage 21±,* in some experiments designed to study certain problems of morphogenesis of the brain. Since it was doubtful whether such individuals could lead an independent existence beyond the yolk resorption stage, 30 embryos were joined parabiotically with normal embryos to serve as nurses, as well as controls for the operated components. The remainder were allowed to develop as "free" individuals. Seventeen cases of parabiotic twins and 9 single individuals survived. In no case was there any external evidence of regeneration of the parts removed.

External malformations in the shape of the head resulting from the ablation are seen in Fig. 1 and 2. The growth of the lower jaw has apparently been unaffected, and is seen to protrude quite far beyond the upper jaw. A detailed study of the morphogenesis of the mid and hindbrain, in the absence of the hemispheres, will be made upon the operated components of the twins, using the brains of the normal components for comparison. A similar comparative study will be made also upon the development of the skull.

When the larvae had reached the feeding stage, 7 of the non-parabiosed individuals, despite the absence of the forebrain, eyes, and nasal organs, exhibited the capacity to feed upon daphnia and small enchytraeid worms. Like normal larvae, they exhibited snapping reactions when a needle, immersed in the water, was moved gently back and forth along side of the head. This capacity to sense moving objects, whether edible or not, bears out former observations,[1] that neither vision nor smell, nor both can be regarded as indispensable to the securing of food. We[1] showed that eyeless larvae, whether reared in light or darkness, grew as rapidly as their controls under similar conditions of feeding. Even when the receptor apparatus was restricted to the lateral-line sense organs, following removal of the eyes and nasal placodes, the growth in young larvae was not impeded. These findings supported the observations of Sharrer[2] who showed that eyeless larvae would snap at minute streams of water directed towards the side of the head. If, however, the primordia of the lateral-line organs were removed from one side, the larvae would snap only if the stimulus were applied to the unoperated side.

Nicholas,[3] who removed the eyes of *A. tigrinum* embryos, claimed that the larvae failed to respond positively to moving objects which did not stimulate the olfactory apparatus. He thus regarded the olfactory apparatus as paramount in obtaining food when the visual sense is obliterated, yet he also recognized that following ablation of both the eyes and nasal organs, the animals were capable of snapping at moving objects, whether edible or not. Previous to the observations of Nicholas, Burr[4] had emphasized the importance of smell, especially after removal of the eyes.

Since the lateral-line sense organs are sufficient for sensing food, the question naturally arises whether, in normal larvae, these organs play a significant role in this capacity.

Food sufficient for *normal* growth, in larval stages can be located by the lateral-line organs alone, but only if the forebrain is in-

* In stage 21, the medullary folds are completely fused.

[1] Detwiler, S. R., and Copenhaver, W. M., *Anat. Rec.*, 1940, **76**, 241.

[2] Sharrer, E., *J. Exp. Zool.*, 1932, **61**, 109.

[3] Nicholas, J. S., *J. Exp. Zool.*, 1922, **35**, 257.

[4] Burr, H. S., *J. Exp. Zool.*, 1916, **20**, 27.

FIG. 1. FIG. 2.

FIG. 1.

Drawing of the case FBEB 44, lacking forebrain, eyes, and nasal organs. 26 days after operation. × 8.

FIG. 2.

Drawing of case FBEB(P) 20, the right component of the parabiotic twin lacks the forebrain, eyes, and nasal organs. The left component is normal. 26 days after operation. × 8.

tact. Larvae lacking the hemispheres are capable of feeding, but their intake is much less and their growth is markedly curtailed. Spontaneous behavior in general is greatly reduced, both quantitatively and qualitatively, especially the foraging activities, regardless of the amount of food available. The lengths of the larvae at 45 days of age are slightly more than 50% of the length of maximally fed normal larvae of similar age.

Nicholas[5] excised the embryonic mesencephalon and replaced it with a limb and pronephric rudiment. Such animals were unable to feed, and he regarded this failure as due to a *block* in the nervous system brought about by mesencephalonectomy. It would appear that if the presence of the mesencephalon is essential, it acts as a coordinator of motor activities rather than serving as a *bridge* between forebrain and medulla, for the present experiments demonstrate that larvae lacking hemispheres are capable of carrying out all activities necessary for obtaining food.

[5] Nicholas, J. S., *J. Exp. Zool.*, 1930, **55**, 1.

The operated components of the parabiotic twins were also seen to snap and engulf food, but in these cases, the eating function was taken over largely by the more active conjoined normal components. Consequently, the growth rate of the twins was greater than in the operated free individuals, but not as great as in normal animals.

Summary. Amblystoma larvae, deprived of the cerebral hemispheres, eyes, and nasal organs react by snapping at inanimate objects in motion. They are capable of feeding upon daphnia and small enchytraeid worms. Their food intake and growth is greatly reduced as compared with these functions in larvae lacking eyes and nasal organs, but with the hemispheres intact.

The lateral-line sense organs alone constitute an adequate receptor apparatus for the detection of food in motion. The motor activities concerned with lurching, engulfing food, chewing and swallowing are carried out in a normal integrated manner, although they are less vigorous than in larvae with intact hemispheres.

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Influence of Diet on Chick Growth-Promoting and Antiperotic Properties of Betaine, Methionine and Choline.*

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Results showing that betaine is effective in preventing perosis and promoting growth in chicks fed a simplified diet (Diet 543) have been reported by McGinnis, Norris and Heuser.[1] The improved growth was found to be highly significant. Jukes and Welch[2] reported that betaine had a growth promoting effect on chicks fed a purified diet, but was without effect in preventing perosis. Almquist and Grau[3] using a purified diet containing isolated soybean protein instead of casein and gelatin reported that betaine and methionine were as effective as choline in promoting growth. In later work Almquist and Grau[4] with a somewhat different basal diet obtained a growth-promoting effect from betaine only when combined with cystine or homocystine. This effect, however, was not equal to that obtained with choline.

In order to explain the discrepancy between the results of McGinnis and associates,[1] Jukes and Welch,[2] and Almquist and Grau,[3, 4] 3 experiments are here reported showing the influence of betaine, methionine and choline on perosis and growth in chicks when added to basal diets differing in composition. Day-old Rhode Island Red cockerel chicks were used in all of the experiments, 12 per lot in Experiment 1 and 15 per lot in Experiments 2 and 3. The experiments were terminated at the end of 4 weeks.

The composition of the experimental diets is given in Table I. Diet 543 was found to contain 0.078% choline, diet 661 to contain 0.027%, and diet 671 to contain 0.040%. The approximate methionine content of diet 661 was 0.85% and the cystine content 0.09%, and the approximate methionine content of diet 671 was 0.38% and the cystine content 0.07%. From a consideration of the available data, it appears probable that the approximate methionine content of diet 543 was 0.55% and the cystine content 0.20%. The data used in obtaining these values were for the most part those reported by Schmidt,[5] Grau and Almquist,[6] and Brown.[7]

The results of the experiments are given in Table II. In Experiment I choline and betaine were used as supplements to diets 543 and 661. The original findings of McGinnis and associates[1] that betaine when added to simplified diet 543 prevents perosis and promotes growth were confirmed. On the other hand, betaine had no effect on perosis or growth when added to purified diet 661. Its ineffectiveness on perosis is in agreement with that obtained by Jukes and Welch,[2] with a purified diet. In contrast to the results with betaine, choline prevented perosis and pro-

* This investigation was supported in part by the establishment of a fellowship at Cornell University by the Cooperative G. L. F. Exchange, Ithaca, N.Y. Grateful acknowledgment is made to Anheuser-Busch, St. Louis, Mo., for the "Strain S" dried brewers' yeast, to Lederle Laboratories, Pearl River, N.Y., for the choline chloride, and to the A. E. Staley Mfg. Co., Decatur, Ill., for the isolated soybean protein used in these experiments.

1 McGinnis, J., Norris, L. C., and Heuser, G. F., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 293.

2 Jukes, T. H., and Welch, A. D., *J. Biol. Chem.*, 1942, **146**, 19.

3 Almquist, H. J., and Grau, C. R., *J. Biol. Chem.*, 1943, **149**, 575.

4 Almquist, H. J., and Grau, C. R., *J. Nutrition*, 1944, **27**, 263.

5 Schmidt, C. L. A., *The Chemistry of the Amino Acids and Proteins*, 1944, 2nd ed., 217.

6 Grau, C. R., and Almquist, H. J., *J. Nutrition*, 1943, **26**, 631.

7 Brown, W. L., *J. Biol. Chem.*, 1942, **142**, 299.

TABLE I.
Composition of Basal Diets.

Ingredient	Diet 543	Diet 661	Diet 671
	g	g	g
Degerminated yellow corn meal	66.75	—	—
Cerelose	—	52.0	52.0
Peanut meal	15.00	—	—
Purified casein	10.00	25.0	—
Isolated soybean protein	—	—	25.0
Gelatin	—	5.0	5.0
Dried brewers' yeast	—	5.0	5.0
Mineral mixture	5.00*	5.0†	5.0†
Corn oil	—	4.0	4.0
Soybean oil	3.00	—	—
Cellophane	—	3.0	3.0
Fortified cod liver oil‡	0.25	1.0	1.0
	mg	mg	mg
Inositol	—	100.0	100.0
p-Aminobenzoic acid	—	10.0	10.0
Niacin	—	3.0	3.0
Pantothenic acid	0.7	1.5	1.5
Alpha-tocopherol	—	1.0	1.0
2-Methyl-1,4-naphthoquinone	—	1.0	1.0
Riboflavin	0.5	1.0	1.0
Pyridoxine	0.5	0.5	0.5
Thiamin	0.3	0.5	0.5
Biotin	—	0.01	0.01

* *Poultry Science*, 1940, **19**, 315.

† Bone meal, 2270 g; K_2HPO_4, 840 g; pulverized limestone, 700 g; NaCl, 600 g; $MgSO_4 \cdot 7H_2O$, 500 g; $Fe_2(SO_4)_3 \cdot XH_2O$, 55 g; $MnSO_4 \cdot 4H_2O$, 29 g; KI, 3.5 g; $CuSO_4 \cdot 5H_2O$, 1.5 g; $ZnCl_2$, 1.0 g; $CoCl_2 \cdot 6H_2O$, 0.2 g.

‡ 2000 U.S.P. units vitamin A and 400 A.O.A.C. units vitamin D per g.

moted growth when added to either diet 543 or diet 661.

In Experiment 2 methionine was used as a supplement to diets 543 and 661 as well as betaine and choline. Again betaine was as effective as choline in preventing perosis and promoting growth when added to simplified diet 543. In agreement with the results of Experiment 1, betaine failed to prevent perosis or promote growth when added to purified diet 661. Methionine was as effective in promoting growth as betaine or choline when added to diet 543 and markedly reduced the incidence and severity of perosis. Like betaine, methionine was ineffective in preventing perosis or promoting growth when added to diet 661.

From a comparison of the results on these diets, it is evident that betaine and methionine when added to diet 543 enable the chick to synthesize choline and that choline is required as such for the prevention of perosis and for growth. The effectiveness of betaine and methionine in preventing perosis and promoting growth when added to diet 543 may be attributed to the presence of an unidentified factor necessary for the synthesis of choline by the chick from these substances. The possibility, however, that a factor which inhibits choline synthesis is present in diet 661, cannot be excluded at the present time.

In experiment 3 additional data were obtained which support the conclusion that choline is required as such for the prevention of perosis and for growth in chicks. Diet 671 fed in this experiment was the same as purified diet 661 except that a fraction of the proteins of soybeans, isolated by special procedures, replaced the purified casein. With possibly one exception the growth response

TABLE II.
Effect of Choline,* Betaine,* and Methionine on Perosis and Growth in Chicks.

Diet	Supplement, 0.2% each	Perosis		Avg wt at 4 wks with sd†
		Incidence %	Severity index	
		Exp. 1.		
543	None	72.5	37.4	195 ± 31.6
	Choline	0.0	0.0	277 ± 46.1
	Betaine	0.0	0.0	266 ± 34.2
661	None	100.0	57.0	151 ± 19.6
	Choline	0.0	0.0	252 ± 72.3
	Betaine	100.0	68.0	150 ± 28.6
		Exp. 2.		
543	None	84.7	38.9	179 ± 59.6
	Choline	0.0	0.0	231 ± 35.8
	Betaine	6.6	0.7	232 ± 45.7
	Methionine	26.6	8.1	227 ± 50.4
	Choline + methionine	0.0	0.0	234 ± 35.7
	Betaine + "	7.7	1.3	205 ± 35.3
661	None	80.0	37.8	187 ± 32.7
	Choline	0.0	0.0	218 ± 55.8
	Betaine	100.0	52.0	174 ± 33.2
	Methionine	93.0	57.2	165 ± 27.0
	Choline + methionine	0.0	0.0	224 ± 68.5
	Betaine + "	93.0	58.8	151 ± 28.3
		Exp. 3.		
671	None	71.4	29.4	82 ± 14.2
	Choline	0.0	0.0	169 ± 30.3
	Betaine	100.0	56.5	124 ± 21.3
	Methionine	100.0	48.8	111 ± 20.2
	Choline + betaine	0.0	0.0	179 ± 33.3
	Choline + methionine	0.0	0.0	210 ± 39.7
	Betaine + "	100.0	56.7	147 ± 27.5
	Choline + betaine + methionine	0.0	0.0	240 ± 40.0

* Added as the chloride.
† Standard deviation.

obtained with choline was approximately the same regardless of whether it was added to diet 671 alone or in combination with betaine, or methionine, or betaine and methionine. Betaine and methionine were effective in promoting growth but not in preventing perosis. The growth results with betaine are in general agreement with those obtained by Almquist and Grau[4] under somewhat different conditions, and by Jukes and Welch[2] using a purified diet of lower methionine content than diet 661.

The discrepancy in the results obtained with diets 661 and 671 is probably due to the fact that the latter diet contained less methionine and hence fewer methyl groups than the former. The methyl-group deficiency is also revealed by comparing the difference in growth response obtained by supplementing those diets with betaine and methionine. Maximum growth and also prevention of perosis on diet 671 were not obtained however, until choline was included in it along with betaine and methionine. The results of this work are in general agreement with those of Almquist and Grau[4] who reported that the growth-promoting effects of betaine or methionine and arsenocholine were additive under their experimental conditions.

Summary. Betaine and methionine have been shown to be effective in preventing perosis and promoting growth in chicks when added to a simplified diet. They were ineffective when added to a purified diet of higher

methionine content. In contrast, choline prevented perosis and promoted growth on both diets.

The results indicate that choline is required as such for the prevention of perosis and for growth and that betaine and methionine when added to the simplified diet enabled the chick to synthesize choline. This may be caused by an unidentified factor present in the simplified diet which is necessary for choline synthesis. Another possible explanation is that a factor which inhibits choline synthesis is present in the purified diet.

When the casein in the purified diet was replaced with an isolated fraction of the proteins of soybeans with resultant reduction in methionine content, betaine and methionine failed to prevent perosis but promoted some growth. The growth response obtained with betaine and methionine in this instance is believed to be due to a deficiency of methyl groups only. Choline was again effective for both purposes.

14651 P

Initial Emptying Time of Stomach in Primigravidae as Related to Evacuation of Biliary Tract.*

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Previous studies[1] have shown that in the second and third trimesters of pregnancy the gall bladder of 13 gravidæ discharged an average of only 49% of contents (within 40 minutes after a standard meal of egg-yolk) as compared with an average discharge of 73% obtained from 12 nulligravidæ; that the best 8 of the 13 discharged an average of only 57% and the remaining 5 an average of only 38%; but when the latter were tested 6 to 8 weeks postpartum, the amount of bile discharged in the 40-minute period (71%) approximated the nulligravid figure.

The present study has attempted to ascertain whether this retardation in rate of emptying of the gall bladder in pregnancy could have been due to a delay in the initial rate of emptying of the stomach. To this end 17 nulligravidæ (nursing students) were examined fluoroscopically after receiving a standard meal of egg-yolk to which 3 heaping teaspoons of barium sulphate had been added. They were then used as controls for 18 primigravidæ of the second and third trimesters, similarly treated. Fluoroscopic observations were supplemented by films. In addition to recording the time of the first spurt into the bulb, and the length of time required for the head of the meal to reach the pars inferior duodeni and the jejunum, an attempt was made to estimate the amount discharged from the stomach during the first 15 minutes. After the above tests were concluded, it was ascertained by experiment that 10 cc of egg-yolk (2/5 of a swallow) was enough to induce evacuation of 72% of the contents of the gall bladder in 35 minutes, when injected directly into the duodenum.

Table I summarizes the most striking results. In each of the 2 groups of subjects there were 13 individuals in whom the pylorus opened within the first few minutes.

* Aided by a grant from the Graduate Research Funds of the University of Minnesota.

1 Gerdes, Maude M., and Boyden, Edward A., *Surg., Gyn., and Obst.*, 1938, **66**, 145.

TABLE I.
Initial Rate of Emptying of Stomach.

Subjects	Time, in minutes, at which meal enters			Estimated amount discharged
	Bulb	Pars inf.		
17 nulligravidæ	Avg of 13 = 2.06 min. (0.25-3.5)	Avg of 11 = 4.04 (1.7-6.8)	9 of 13 = at least 10 cc (1-17 min.)	
	" " 4 = 14.8 " (7.2-20)	" " 2 = 35+	4 of 13 = " " 5-7 " (4-13)*	
		" " 4 = 19.8 (17.2-22.8)		
18 primigravidæ	" " 13 = 2.72 " (0.3-8.7)	" " 7 = 3.43 (0.6-6.8)	8 of 13 = " " 10 cc (1.2-14 min.)	
		" " 3 = 24.95 (16.7-29.5)	5 of 13 = " " 3-5 cc (1-10) †	
		" " 3 = 33+		
	" " 5 = 32 " +	" " 5 = 33+		

* 2 cases, no record after 6 min. † 1 case, no record after 7 min.

(This corresponds to the period in which the gall bladder first contracts.) The other 4 or 5, in each group, showed a more or less delayed reaction. It took longer, however, for the head of the meal to reach the inferior angle of the duodenum in more of the first 13 primigravidæ than in the corresponding controls. This suggests that in pregnancy there is a slowing down of peristalsis. But since there was sufficient food in the duodenum to empty the gall bladder in a comparable number of cases in each group, it would not appear that the rate of emptying of the stomach is a primary factor (if one at all) in causing delayed evacuation of the gall bladder in pregnancy.

Recently two groups of workers[2,3] conducting experiments with guinea pigs, have presented interesting evidence that female sex hormones diminish the contractile force of the gall bladder. This finding is in accord with the observation noted in the first paragraph of this article—namely, that even the best 8 of the gall bladders studied in pregnancy evacuated much more slowly than the controls. However, it may be questioned whether the negative findings of these authors, with respect to the sphincter of Oddi, can be applied to human gravidæ: first, because the sphincter of the guinea pig (unlike that of man) is a peristaltically active evacuating mechanism (the functional rate of which, incidentally, was not tested in the above mentioned experiments) and secondly because the evidence seems to indicate that in the human blood stream hormones that affect the musculature of the gall bladder likewise affect the sphincter.[4]

[2] Smith, J. J., Pomarane, M. M., and Ivy, A. C., *Am. J. Physiol.*, 1941, **132**, 129.

[3] Myers, G. S., and Hill, W. T., *Am. J. Physiol.*, 1942, **135**, 347.

[4] Boyden, Edward A., Bergh, George S., and Layne, John A., *Surgery*, 1943, **13**, 723.

14652

Effects of Thiouracil and Sodium 5, 5-Diphenyl Hydantoinate (Dilantin Sodium) on Resistance to Lowered Barometric Pressures.*

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Attempts have been made recently to increase the resistance of animals to reduced barometric pressures by the administration of certain chemicals. Thiourea[1,2] and thiouracil[3,4] have been found to be effective in this respect.

Such drugs probably act by preventing the formation of thyroid hormone[5,6] thereby increasing the ability of the animal to tolerate the ill-effects of anoxia. It had been previously demonstrated that surgical thyroidectomy is beneficial[7,8,9] and treatment with thyroid or anterior pituitary substance harmful[7,8,10,11] to animals exposed to oxygen lack.

Another drug shown to protect rats and mice subjected to anoxia is dilantin.[12,13,14] Hoff and Yahn[14] have suggested the possibility that this drug operates by stimulating directly the activity of the nerve cells in the respiratory and cardiac centers.

It was considered important to compare the effects of thiouracil and dilantin and also to determine the action of the combination of these drugs on the resistance of rats to reduced barometric pressures.

Methods. Sixty-eight adult male rats weighing 190-250 g were employed. Half of these were fed a stock diet containing 0.2% thiouracil‡ for 3 weeks. Four groups of 17 animals were then established as follows: One group of the thiouracil-fed rats received intraperitoneal injections of 15 mg dilantin sodium‡ approximately 1½ hours before exposure to the reduced barometric pressures. Another group, consisting of controls fed ordinary stock diet, likewise received 15 mg injections of dilantin prior to exposure. The remaining 2 groups comprised thiouracil-fed rats given no additional treatment, and a series of untreated controls. Actually 2 experiments were conducted at 2 different times but because of the similarity in the results obtained, the data will be presented in pooled form.

All animals were subjected to a pressure of 148 mm Hg (39,000 feet) in a specially constructed low pressure chamber equipped with a neon light and several observation windows. The rats were brought to this pressure over a period of 20 minutes. Once the reduced pressure was established, the time for complete cessation of respiration in each animal was recorded. The pressure was maintained at 148 mm Hg for 2 hours. The remaining living animals were then brought to a pressure of 92 mm Hg (49,000 feet) over a period of 20 additional minutes.

* Supported by a grant from the Commonwealth Fund.

† Also of the College of the City of New York.

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13 Emerson, G. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 252.

14 Hoff, E. C., and Yahn, C., *Am. J. Physiol.*, 1944, **141**, 7.

‡ We wish to thank Dr. B. W. Carey of the Lederle Laboratories, Inc., for the thiouracil and Dr. E. C. Vonder Heide of Parke, Davis and Co. for a supply of dilantin sodium.

TABLE I.
Action of Thiouracil and Dilantin on Resistance to Low Barometric Pressures.

Treatment	Mean survival time (min.) ± standard error	Mean thyroid wt (mg) ± S.E.	Avg degree of lung hemorrhage %
Thiouracil	34.8 ± 6.0	73.3 ± 4.8	67.6
Dilantin	32.1 ± 4.2	17.0 ± 0.38	54.4
Thiouracil + Dilantin	> 120*	60.0 ± 3.5	10.3
None	9.6 ± 1.0	16.8 ± 0.43	72.1

* 14 out of 17 rats in this group survived the 2-hour exposure to 148 mm Hg (39,000 feet). They succumbed when the pressure was lowered to 92 mm Hg (49,000 feet).

At the termination of the experiment all animals were autopsied, the thyroids and adrenals dissected, and weighed to the nearest milligram on a torsion balance. The eyes were examined for the development of lens cataract known to occur under conditions of anoxia[15] and the lungs for signs of hemorrhage. The lung condition was judged as 0, +, ++, +++, or ++++ depending on the severity of the damage, according to the description given by Lawson, Carnes, and Thienes.[16] The average degree of hemorrhage was computed by adding the number of "plusses" and dividing this total by the number of animals in the group. This figure was then multiplied by 25, giving the extent of hemorrhage as per cent.[16]

Results. The data presented in Table I confirm the previous findings[1,2,3,4,12,13,14] that thiouracil and dilantin are capable of increasing the resistance of rats to lowered barometric pressures. These drugs, in the dosages employed, appear to be equally active in producing this effect. It is also quite apparent that the combination of the 2 drugs is far more effective than either one alone.

The results indicate that the combination of drugs also affords considerable protection to the lung tissue. Whereas the greater majority of the untreated controls and those given only thiouracil display marked lung damage at autopsy, those thiouracil-fed rats which are given dilantin prior to exposure show either very little hemorrhage or none at all. A slight protective action also appears to have been exerted by dilantin itself. It does not seem likely that prevention of lung damage is directly accountable for the increased survival time since it has been found in both the present and in unpublished experiments that thiouracil or thiourea acting alone can considerably increase resistance to decompression without rendering any protection to the lung.

The marked increase in thyroid weight in animals receiving thiouracil has already been described at length.[17,18] This goitrous condition is associated with a state of functional hypothyroidism and, in the present experiments, was apparent in every rat given the drug in their diet. Adrenal weights, not included in Table I, were not significantly altered by any of the experimental treatments. Lens cataract which developed in a considerable number of animals subjected to decompression was not prevented by the drugs given singly or in combination.

The striking synergism between thiouracil and dilantin which probably act through different mechanisms to increase resistance to lowered pressures, is one of considerable interest and should be studied more intensively.

Summary. 1. Both dilantin and thiouracil prolong the survival time of rats subjected to reduced barometric pressures (148 mm Hg). 2. The combination is considerably more effective than either one acting alone. 3. The combination tends to reduce the extent of lung hemorrhage in rats subjected to decompression but has no effect on the development of lens cataract.

15 Bellows, J. B., and Nelson, D., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 126.

16 Lawson, H. C., Carnes, H. O., and Thienes, C. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 327.

17 Mackenzie, C. G., and Mackenzie, J. B., *Endocrinology*, 1943, **32**, 185.

18 Astwood, E. B., Sullivan, J., Bissell, A., and Tyslowitz, R., *Endocrinology*, 1943, **32**, 210.

14653 P

High Resistance of Rhesus Monkeys to 90 Plus Per Cent Oxygen.

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The use of very high oxygen concentrations for clinical purposes has for many years been restricted by the inability of the lower mammals to live safely in continuous oxygen concentrations exceeding 70%. Barach[1] has shown that rabbits can live in 60% concentrations even as long as 3 months. He[2] considers 70% the safe top level for them. Oxygen mixtures between 80 and 100% produce irritant inflammatory lesions in the lungs of rabbits, dogs, cats, mice, rats, birds and other small laboratory animals, when used continuously for 2 to 5 days.[3a, 3b] The animals employed in these experiments had high metabolic rates and rapid respiratory rates. Rabbits have a respiratory rate of about 100 to 150 per minute. It is interesting to note that turtles and frogs,[3b] with extremely slow respiratory rates, are able to live safely in oxygen concentrations of 90+% for months, when the temperature is maintained at 70° F. However, when the temperature of the oxygen is raised to 99.5° F, they develop pulmonary edema. The Rhesus monkey[4] with a normal rate of 24 to 40 gives us an animal with a respiratory mechanism anatomically and physiologically more closely resembling the human. With one exception, in which one monkey was employed, no workers have used Rhesus monkeys in their experiments. In our work 14 rabbits were used as controls, 2 at a time being exposed to the same atmospheres as the monkeys.

Experiments. Four Rhesus Monkeys and 2 rabbits were placed in a Barach-Thurston oxygen tent containing soda-lime, with a specially sealed canopy, and the concentration was raised to 90-95% and maintained at this level continuously for 20 days. Every 4 days 2 rabbits died and were replaced by 2 new controls. On the fourth day the monkeys developed slight anorexia. On the fifth day one of them coughed and developed dyspnea. On the sixth day all 4 monkeys appeared bright again and regained their appetites. On the eleventh day 3 monkeys were active and eating well. On the sixteenth day the first monkey died. On the eighteenth day another died. After 20 days the concentration was gradually reduced and 2 monkeys, apparently healthy, were removed. Roentgenograms of the chest were checked. One was entirely negative and the other showed 2 small areas of infiltration.

Eight Rhesus Monkeys (6 new ones and 2 survivors of the previous experiment) and 4 rabbit controls (two at a time every 4 days as in the previous experiment) were placed in 90-95% oxygen atmosphere. One monkey coughed on the third day, all appeared well and active on the fourth day. On the fifth day they developed anorexia, were apathetic, and a few of them had labored breathing. On the sixth day one died; on the seventh another died and 4 were active and normal. Two more died on the eighth day and 2 on the tenth day. Two were removed in good health and remained well. These 2 monkeys were survivors from the first experiment. During this experiment the tent temperature frequently dropped to 60°. In both experiments oxygen and carbon dioxide analyses were performed 5-15 times daily. Humidity per cent and tent temperature were simultaneously observed.

Autopsy Findings. The significant findings at autopsy were confined to the lungs. The other organs were normal and showed only passive congestion.

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2 Barach, A. L., *N. Y. State Med. J.*, 1937, **37**, 1095.

3 a. Karsner, H. T., *J. Exp. Med.*, 1916, **23**, 19; b. Binger, C. A. L., Faulkner, J. M., and Moore, R. L. J., *J. Exp. Med.*, 1927, **45**, 849.

4 Hartman, C. G., and Strauss, Wm. L., *The Anatomy of the Rhesus Monkey*, Williams & Wilkins, Baltimore, 1933.

In the rabbits the process in the lungs was diffuse and involved all the lobes. The surfaces were purple red except at the margins where the tissue was pink and crepitant. The lobular markings were distinct. The bronchi and bronchioles contained a pink frothy fluid and the cut surfaces were wet. Scattered throughout were numerous granular raised areas. Microscopically the lung preparations showed extensive congestion and edema with numerous foci of pneumonia.

In the monkeys the process was patchy and far less extensive. The greater portion of the lungs were pink and air-containing. The cut surfaces, however, were moist and the bronchioles contained some pink frothy fluid. Some granular areas were also noted, particularly in the lower lobes. Microscopically the lungs showed congestion with some edema and a few small foci of pneumonia. There were also some small areas of emphysema.

Summary and Conclusions. Ten normal Rhesus Monkeys continuously exposed to 90+% oxygen appeared to maintain good health for a 5-day period. Then the reaction was variable. Anorexia first appeared, then apathy, drowsiness, cough and dyspnea. Survival periods ranged from 6 to 18 days in 8 monkeys. Two were alive at the end of 20 days and remained in good health. Pairs of rabbits used as controls died after 4 days and were replaced by new controls who again died after 4 days. Autopsies were performed and showed a slight amount of congestion and exudate in the lungs resembling a mild pneumonia in the monkeys, while the rabbits showed extensive pneumonia. The marked difference in reaction of the two sets of monkeys suggests that there may be a difference of resistance to 90+% oxygen in different strains, or that lowering the tent temperature to 60° may have played a role in diminishing their resistance. The reaction of the Rhesus Monkeys suggests the possibility that it may be safe for human beings to be exposed continuously, if necessary, to a 90-95% oxygen concentration for periods up to 5 days.

14654

Studies on the Action of Penicillin. III. Bactericidal Action of Penicillin on Meningococcus *in vitro*.

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The meningococcus is highly susceptible to penicillin although it is gram-negative. In this respect as in its reaction to certain germicides, it resembles the gram-positive bacteria.[1] A preceding communication[2] reports a study of the action of penicillin on meningococcus *in vivo*.

Methods. Suspensions of meningococci grown for 6 hours on agar slants were made up in gelatin-Locke's solution or broth to contain approximately 10^8 microörganisms per ml. After the addition of other ingredients the tubes were slanted on a rack and incubated in that position to expose the maximum surface to the air. Samples were withdrawn at frequent intervals and 10-fold dilutions made in gelatin-Locke's solution. Loopfuls were cultured from appropriate dilutions by careful streaking onto the surface of blood agar plates. Each bacterial count was based on 3 platings. Repeated checks on the accuracy of this method showed it to be adequate for the purposes at hand and better for meningococci than the ordinary methods involving the use of poured plates. Plate cultures were examined after 24 hours' incubation in

1 Miller, C. P., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 197.

2 Miller, C. P., and Foster, A. Z., PROC. SOC. EXP. BIOL. AND MED., in press.

a candle jar and again after 48 hours.

Preliminary experiments had demonstrated that 10 units of penicillin* per ml of broth could reduce the number of meningococci from 10,000,000 to 100,000 per ml in 2½-3 hours. Within the range at which we worked (10 units per ml) 10-fold differences in concentration of penicillin made but unimportant changes in the results.

Effect of Fresh or Inactivated Serum on the Activity of Penicillin in Broth. Serum was obtained from normal snuffle-free rabbits and a part of it was inactivated for 30 minutes at 56°. The fresh serum added in concentrations of 10-50% to broth containing 10 units of penicillin per ml speeded up the rate of decrease in the number of viable meningococci. That this gain in meningococcidal action was due to a heat-labile substance (complement) was demonstrated by comparing the effect of inactivated serum, which not only failed to accelerate the decrease in numbers of meningococci as compared with the broth, but in some experiments slowed it down to an appreciable degree. (Fig. 1 and 2.)

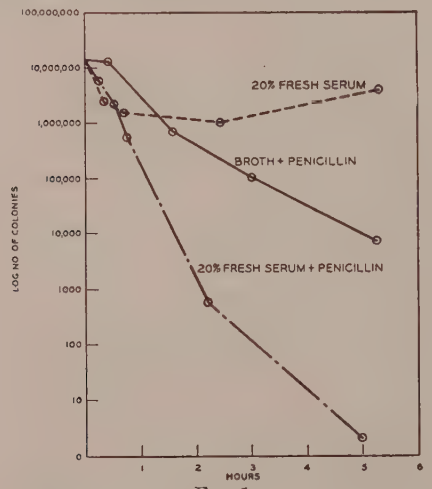

FIG. 1.

Effect of fresh normal rabbit serum on the meningococcidal action of penicillin.

Broth containing fresh rabbit serum without penicillin caused an abrupt but transitory decrease in the population which was followed by a rise. It seemed most likely, therefore, that the greater activity of penicillin in fresh normal serum was a summation of 2 meningococcidal agents rather than an interaction of penicillin with some heat-labile substance in the serum.

Within certain limits the extent of the meningococcidal action of fresh normal rabbit serum was proportional to its concentration. The results with sera from different rabbits, while not identical, fell within limits which might be expected from individual variation. The fresh and inactivated serum of man, guinea pigs, and mice gave results similar to those with rabbit serum. Not all strains of meningococcus were equally susceptible to fresh serum.[3]

The Effect of Penicillin in Non-Nutrient Medium. Preliminary observations demonstrated that the viable population of meningococci suspended in gelatin-Locke's solution

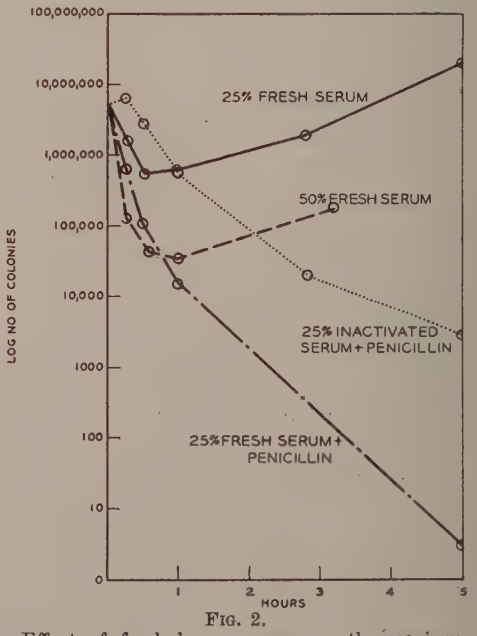

FIG. 2.

Effect of fresh human serum on the meningococcidal action of penicillin.

* The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for experimental investigations recommended by the Committee on Chemotherapeutics and Other Agents of the National Research Council.

[3] Thomas, L., and Dingle, J. H., *J. Clin. Invest.*, 1943, **22**, 375.

TABLE I.
Effect of Temperature on Action of Penicillin.
Colonies per ml developing in subcultures from broth suspensions.

	Broth	Broth containing 10 units penicillin per ml
After 24 hr in ice box	10,000,000	11,000,000
After 48 hr in ice box	5,300,000	6,300,000
Transferred to incubator, examined 18 hr thereafter	∞	0
		Washed sediment from 2 ml

remained practically constant (within the limits of accuracy of the method) for a period of 6 hours at 37°. The addition of penicillin to such suspensions was almost without effect on the bacterial population. (Fig. 3.)

When inactivated rabbit serum was added in a concentration (10%) which was able to promote some multiplication of meningococcus in the otherwise non-nutrient medium, penicillin reduced the population about as rapidly as it did in broth.

This observation supports the contention of Hobby, Meyer, and Chaffee,[4] that penicillin acts only under conditions favorable to bacterial multiplication.

Effect of Temperature on the Action of Penicillin in Broth. Ten units of penicillin per ml were added to one half of a broth suspension of meningococci and both tubes placed in the ice box. Samples were withdrawn after 24 and 48 hours, diluted and plated as in the previous experiment. The results of a typical experiment are presented in Table I and show that exposure to this concentration of penicillin for 48 hours caused no decrease in numbers of meningococci. When these same suspensions were transferred to the incubator for 18 hours, heavy growth occurred in the broth control and the penicillin suspension became sterile.

Bacteriolysis. Lysis by penicillin could be observed if the suspensions contained approximately 2-5 x 10^8 per ml meningococci which gave the solution a distinct haziness, but not if the bacterial suspensions were much heavier than this. Ten units of penicillin per ml produced definite, but incomplete clearing which could be increased only by great increases in concentration.

Lysis occurred at a rate which was barely

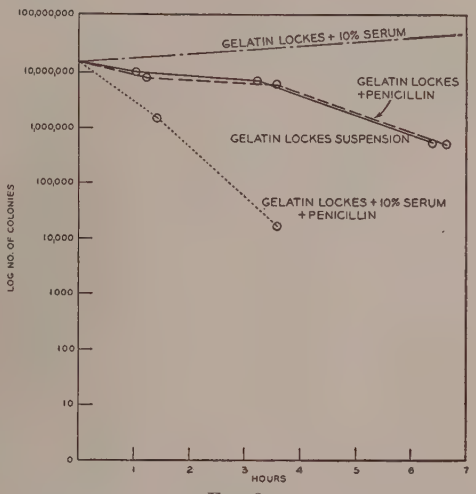

FIG. 3.
Ineffectiveness of penicillin upon meningococci in a non-nutrient medium.

detectable from hour to hour, but was quite distinct at the end of 24 hours after which little or no change could be noted. It was most pronounced at 37°, although it progressed more rapidly at 45° for the first hour, and then stopped altogether. Lysis did not occur at ice box temperature.

Meningococci killed by heating at 45° for 15 minutes were not lysed by penicillin, nor were meningococci recovered from old (48-hr) cultures.

These observations on meningococcus do not agree with the findings of several investigators who report failure to demonstrate lysis of other bacteria.[4, 5]

Morphological Changes. In dilutions of penicillin beyond the concentration which inhibited the growth of meningococcus, changes occurred in the morphology of some cells which could be plainly seen in ordinary micro-

[4] Hobby, G. L., Meyer, K., and Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 281.

[5] Waksman, S. A., *J. Bact.*, 1943, **45**, 64.

scopic preparations. Enlargement and distortion from normal shape were the most frequent abnormalities. Such changes were not found by Gardner[6] in meningococci.

Summary and Conclusions. Meningococci are readily killed by penicillin in nutrient broth at 37°. This meningococcidal action is markedly increased by fresh serum of man, rabbit, guinea pig and mouse, due to a heat-labile component, presumably complement. The meningococcus therefore is an unsuitable microorganism on which to test human sera for their content of penicillin by the bacteriostatic method commonly employed. The presence of inactivated serum, however, frequently reduces the activity of penicillin on meningococci in broth.

Penicillin fails to kill meningococci in a non-nutrient medium (gelatin-Locke's solution) at 37° and in nutrient broth at ice box temperature, two conditions unfavorable for their growth. These findings lend support to the notion, already expressed by others, that penicillin acts on bacteria only in an environment which promotes their multiplication.

Penicillin causes a certain degree of lysis of meningococci actively growing in broth.

Meningococci in broth containing concentrations of penicillin just below the level of bacteriostasis develop enlarged forms which suggest an inability to complete the normal process of fission.

[6] Gardner, A. D., *Nature*, 1940, **146**, 837.

14655

Effect of High Protein (Meat) Diet on Mortality from Surgical Shock Due to Repeated Hemorrhage.*

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A high protein diet consisting of horse meat *ad libitum* was given to dogs later subjected to repeated hemorrhage according to a standardized technic previously described.[1] A definitely beneficial influence was observed as compared with animals not so prepared. Although there is a considerable literature on the effect of diet on the regeneration of serum protein from the low concentrations produced by non-fatal hemorrhage, we could find no studies on the influence of the preceding dietary regime on an acute fatal bleeding.

Experimental Procedure. Fatal surgical shock was produced by the removal of 10 cc per kilogram of body weight each hour through puncture or cannulization of the femoral artery, under local anesthesia. The supine position was maintained during the entire procedure. Mongrel dogs were used weighing from 7 to 12 kg. Four groups of experiments were performed as follows:

Group 1 consisted of 14 animals who had been on a non-protein diet[2] for 4 of 5 weeks; during the fourth week various forms of protein were given by mouth to determine their effects on serum protein regeneration. The average caloric intake was 50 per kilo per day.

Group 2 consisted of 10 animals who had been on a "normal" ration of Purina chow for periods of 1 to 3 weeks before the experiment. The approximate proportions of protein, fat, and carbohydrate in the Purina chow are 4, 1, and 9 respectively. The average caloric intake was not measured in the group.

Group 3 consisted of 10 animals given an *ad libitum* horse meat diet for a period of 3 weeks before the experiment was carried out. The amount of meat consumed daily in this

* Aided by a grant from the Commonwealth Fund.

[1] Elman, R., and Lischer, C. E., *Ann. Surg.*, 1943, **118**, 225.

[2] Sachar, L. A., Horvitz, A., and Elman, R., *J. Exp. Med.*, 1942, **75**, 453.

TABLE I.
Survival Periods After Hemorrhage.

Hours	No. surviving 0	1	2	3	4	5	6	Avg survival hr
Depleted	14	14	14	9	2	0	0	3.3
Normal	10	10	10	7	6	0	0	3.7
3 wks horse meat	10	10	10	10	8	0	0	4.5
5 " " "	10	10	10	10	9	5	0	4.9

and the next group averaged 10% of the body weight. No other food was ingested. Analysis of the meat showed a content of about 20% protein (N × 6.25) and 4% fat. The average caloric intake was 120 per kilo per day.

Group 4 consisted of 10 animals given the same diet as in Group 3 for a period of 5 weeks before the experiment.

Experimental Findings. The data observed are described in Table I. It will be observed that the average survival time was but 3.3 hours in animals who had been previously on a non-protein regime, that the figure was 3.7 hours in the "normal" group, but that the animals on horse meat for 3 weeks survived 4.5 hours, whereas those on the same diet for 5 weeks survived 4.8 hours. That these averages are significant can be appreciated by consulting the number of animals actually surviving at each hour.

Comment. It seems clear from the evidence herein presented that dogs on a high protein diet consisting entirely of horse meat for 3 and 5 weeks are able to withstand significantly greater loss of blood than those not so prepared. While it is possible that the beneficial effect of the horse meat diet may be due to high protein content, some other factor or factors may have been responsible for the results. For example, experiments have been described[3] indicating the beneficial effect of thiamine in hemorrhage; however, horse meat has been reported as containing very little thiamine.[4] Regardless of the explanation, there would seem to be a practical inference from these experiments, *i.e.*, that the ingestion of a high protein diet of meat might be advisable as a prophylactic measure in patients in whom severe repeated blood loss may be expected.

Summary. Dogs given an *ad libitum* diet of horse meat for 3 and 5 weeks before repeated fatal hemorrhage are able to withstand a larger amount of blood loss than those on a "normal" or on a non-protein diet.

[3] Govier, W. M., *J. Pharm. and Exp. Therap.*, 1943, **77**, 40.

[4] Pyke, Magnus, *Biochem. J.*, 1940, **34**, 1341.

14656

Muscle Pain, Tendon Reflexes, and Muscular Coordination in Man.*

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The muscular action resulting from cortical innervation is greatly modified by afferent impulses. This is demonstrated by the inability of a monkey to use the hand after denervation of its posterior roots.[1] Moreover, distinct effects may be exerted on the cerebral cortex not only when the normal afferent impulses are absent, but also when they are increased as shown by observations of Uchtomsky.[2] It appeared, therefore, not un-

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Mott, F. W., and Sherrington, C. S., *Proc. Roy. Soc.*, 1895, **57**, 481.

[2] *Cf.* Ufland, J. M., *Pflüger's Arch.*, 1925, **208**, 87.

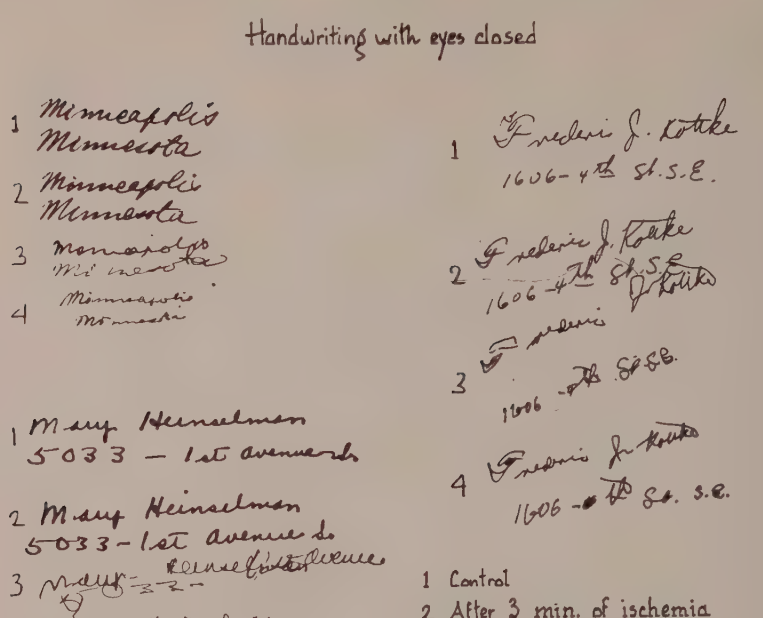

FIG. 1.

likely that pain originating in various peripheral structures may have a marked influence on the effects of central innervation. This problem was studied with respect to muscle pain which was produced by Lewis'[3] method of ischemic pain. Handwriting with closed eyes was used as an indicator of muscular coordination; in addition, the influence of muscle pain on tendon reflexes was studied.

Ischemia of the arm was produced by raising the pressure in a blood pressure cuff above the systolic blood pressure. Three minutes afterwards a writing test was performed in order to determine the effect of ischemia on muscular coordination. Then the fingers were alternately flexed and extended at a rate of 40 to 60 per minute for 40 to 60 seconds until the "end point" was reached. The muscle pain showed the diffuseness mentioned by Lewis. Its occurrence was indicated objectively by the appearance of a flushed face. While the pain persisted after the cessation of the movements the writing test was repeated with closed and also with open eyes. Then the pressure was released and the pain disappeared almost instantaneously.

Fig. 1 illustrates the effects on 3 subjects. It shows clearly that ischemia, at least within the time limits of our experiments, had no effect on handwriting. However, muscle pain initiated in the ischemic muscle by contractions lasting only 40 to 60 seconds causes a profound alteration in writing (*cf.* No. 3 in Fig. 1). These changes persist to a varying degree when the test is repeated with open eyes. The fact that there is an improvement in handwriting when the eyes are open distinguishes this form of disturbance of muscular coordination from that seen in anoxia[4] where the evaluation of optical sensations as well as of those originating in other sense organs is impaired.

As a further indication of disturbance in muscular coordination the finger-finger, and finger-nose tests were employed. It was found that the former was distinctly impaired during

[3] Lewis, T., *Pain*, N.Y., 1942.

[4] Gellhorn, E., *Am. J. Psychiat.*, 1939, **93**, 1, 413.

ischemic pain in 3 out of 6 tests, and the latter in 6 out of 8 tests. It is worthy of mention that in some instances repetition of the same test led to some improvement in spite of the persistence of pain.

Experiments to be reported elsewhere on action potentials in the human and on the effects of stimulation of the motor cortex under the influence of muscle pain give evidence that muscle pain produces changes in the reactivity of the motor cortex. It seems to be probable that these central changes are linked up with the disturbances in muscular coordination reported in this paper. Moreover, there is further proof of changes in the human central nervous system following muscular pain as shown by the following experiments.

The effect of muscle pain on the triceps reflex was studied in 10 persons in whom this reflex was very vivid. It was found that ischemia maintained for several minutes did not alter the reflex, nor had muscle pain, originating in the biceps through repeated contractions of this muscle during ischemia, any effect on the triceps reflex. However, when pain was elicited in the triceps by repeated contraction of the ischemic triceps, the triceps reflex disappeared completely even when stronger stimuli were applied to the tendon than under control conditions. These results were uniform in all 10 subjects. After restoration of the circulation the pain disappeared instantaneously and the triceps reflex returned within a few minutes. In some instances the reflex was fully restored after 30 seconds. In other cases it took one to 3 minutes. During this time a gradual increase in the responsiveness of the reflex was observed. This effect of muscle pain was absent in the contralateral triceps.

That the inhibition of tendon reflexes is due to pain and not to circulatory changes is indicated by the fact that ischemia lasting several minutes does not alter the triceps reflex. Moreover, the reflex remains inhibited immediately after restoration of circulation although the blood flow through the previously ischemic limb is greatly accelerated at this time (Lewis, Pickering, and Rothschild).[5]

[5] Lewis, T., Pickering, G. W., and Rothschild, P., *Heart*, 1931, **15**, 359.

The existence of nerve fibers originating in muscle which on stimulation lead to a reflex relaxation of this particular muscle was demonstrated by Sherrington.[6] These fibers may well be excited by pain and cause the tendon reflex to be absent since it is known that when the extensors are relaxed as under conditions involving reciprocal innervation tendon reflexes cannot be elicited. The fact that the painful triceps does not call forth reflex changes in other muscles such as the biceps is not surprising since stimulation of proprioceptive fibers produces a reflex only in the stimulated muscle and not in large groups of muscles which may be excited via cutaneous receptors. However, with increasing severity muscle pain may affect large areas of the motor cortex as will be shown elsewhere.

That muscle pain abolishes voluntary innervation is common experience; that it may lead to reflex inhibition was suggested by Sherrington.[6] However, no systematic studies seem to have been made previously on this subject. In view of the similarity of the symptoms produced by pain in various deep structures such as muscle, tendon, periosteum, etc. (Lewis)[3], a chance observation of Hess[7] that the muscle of a painful fractured leg failed to shiver on exposure to cold is of interest in that it likewise seems to demonstrate that pain originating in deep structures may inhibit the anterior horn cells. Inhibitory effects of pain on autonomic centers are well established. Ury and Gellhorn[8] showed that pupillary dilation following painful stimulation is due solely to an inhibition of the parasympathetic tone.

Summary. Muscle pain induced by 40 to 60 contractions of the ischemic muscle of the hand causes a severe disturbance in the coordination of voluntary movements as shown by the handwriting and finger-nose tests. That reflex movements are likewise altered is shown by the fact that the triceps reflex disappears regularly when ischemic pain is produced in this muscle. However, neither

[6] Sherrington, C. S., *Nature*, 1924, **113**, 929.

[7] Hess, W. R., *Pflüger's Arch. Ges. Physiol.*, 1924, **203**, 539.

[8] Ury, B., and Gellhorn, E., *J. Neurophysiol.*, 1939, **2**, 268.

ischemia nor ischemic pain involving the biceps has any influence on the triceps reflex. It is concluded that muscle pain interferes with muscular coordination through influences on spinal and possibly cortical centers.

14657

Pharmacological Observations on Crystalline Sodium Penicillin.

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Through the kindness of Dr. O. Wintersteiner, of the Division of Organic Chemistry of this Institute, a small quantity of twice-crystallized Na Penicillin-G was made available for pharmacological experiments which had to be restricted owing to the great scarcity of this pure antibiotic. Its potency was 1650 Oxford units per mg.

Results. Hemolysis of washed rabbit erythrocytes suspended in 0.15 M NaCl or in 0.15 M Na penicillin was compared at 37° after the addition of one part of erythrocyte-suspension in isotonic saline equivalent to original blood to 10 parts of a solution of NaCl or of Na penicillin. No hemolysis occurred during 3 hours. After 17 hours, the erythrocytes suspended in Na penicillin solution had become converted into a chocolate-colored granular mass not easily homogenized; the suspending fluid was water-clear. Microscopically the brown material appeared to consist of granules about 0.5 micron in diameter; a few irregular "ghosts" could be found. No further change occurred during a total period of 48 hours after which only 4% of the original antibiotic activity remained in the supernatant fluid.

Dr. R. J. Dubos suggested that possibly adsorption of penicillin on the rabbit erythrocytes had occurred. In another experiment, the suspending fluid contained 1 mg (1650 units) of Na penicillin per ml together with sufficient NaCl to make the solution equivalent to 0.15 M NaCl. Erythrocyte suspensions with and without penicillin as well as Na penicillin-NaCl solutions without erythrocytes were incubated at 37° for 2 hours in duplicate. There were suitable control tubes from which erythrocyte suspension in saline or the supernatant fluid of such a suspension was added to incubated Na penicillin solution at the time of the antibiotic tests. No hemolysis occurred. Within the limits of error (± 20%) of the determination of antibiotic activity, which was kindly estimated by Miss C. M. McKee, there was no loss of activity in any tube including those from which the erythrocytes had been removed by centrifugation at the end of the period of incubation. It appears unlikely that important amounts of penicillin are adsorbed by rabbit erythrocytes suspended in isotonic saline solution.

Dr. Eric G. Ball, of Harvard Medical School, performed a careful experiment to determine whether yeast cells, duck erythrocytes, or rat liver slices consume oxygen at an altered rate if 3400 units (2.05 mg) of Na penicillin replace an osmotically equivalent amount of NaCl in each ml of Locke's solution containing phosphate buffer at pH 7.33 and glucose as the substrate. All observations were made at 37° C. The solution of Na penicillin was added to the suspensions after a control period of 20 minutes. The oxygen-consumption of other control suspensions was followed throughout the experiment. The total period of observation was 214 minutes. The Na penicillin caused no change in any of the 3 tissues with regard to the rate of oxygen-consumption.

The results of representative tests with a few isolated organs are illustrated in Fig. 1-3. A detailed description of the individual trac-

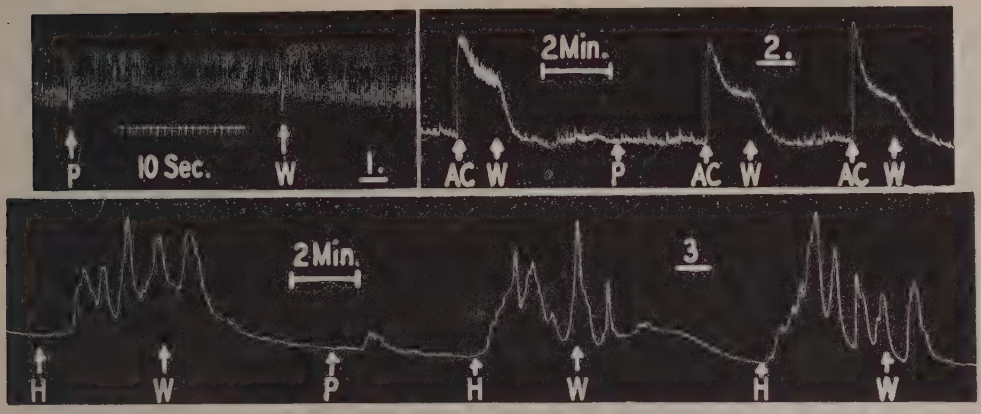

FIG. 1. Effect of Na penicillin on the isolated frog heart perfused by Straub's method. Control perfusing fluid contained 0.001 M $CaCl_2$, 0.002 M KCl, 0.003 M $NaHCO_3$, and 0.111 M NaCl. At P the control fluid was replaced by one containing 0.111 M Na penicillin but otherwise identical. At W the control fluid replaced the solution containing penicillin. The only noteworthy change was a decrease in the amplitude of contraction.

FIG. 2. Isolated guinea-pig ileum. Bath fluid: solution of van Dyke and Hastings containing 0.75 mM $CaCl_2$. At AC, acetylcholine Cl was added to final concentration of 1 to 6.67×10^8; at W the preparation was washed without exposure to air; at P, Na penicillin was added to a final concentration of 1 to 5.46×10^3. Note that the Na penicillin neither caused any change in spontaneous movements nor interfered with the response to acetylcholine Cl.

FIG. 3. Isolated virgin guinea-pig uterus. Same bath fluid as in Fig. 2. At H, an extract of U.S.P. standard posterior-lobe pituitary powder was added in a concentration equivalent to 1 part of powder to 10^8 parts of bath fluid. At P, Na penicillin was added to a final concentration of 1 to 5.46×10^3. W indicates washing without exposure to air. A trivial uterine contraction followed the addition of Na penicillin which did not alter the uterine response to posterior pituitary extract.

ings is given under the figures. These tests confirm the generally-held view that penicillin has a remarkably low toxicity.

The limited quantity of Na penicillin available permitted only a few toxicological observations in mice. Male mice which weighed 21.6-24.6 g initially were used. Na penicillin was injected intravenously as an isotonic solution in comparison with control injections of isotonic saline (both 0.15 M.) over a continuous period of about 20 minutes without anesthesia. Single doses of 1,690,000-1,770,000 units per kg (2 mice) were without apparent effect on behavior, body weight, hemoglobin, total white and red cell counts. Serial electrocardiographic tracings were made before, during and after intravenous injection of 3,820,000 units per kg during a period of 21 minutes. Barbital solution (pH 8.4) was used to produce anesthesia (15 ml of 0.02M solution per kg intraperitoneally); 6 hours later 0.6 mg of picrotoxin per kg was administered subcutaneously to facilitate recovery. A control mouse was similarly treated except that a similar volume of isotonic NaCl instead of isotonic Na penicillin was injected intravenously. (Another control mouse was killed after tracings had been made.) The heart rates changed as follows:

Solution injected	Heart beats per minute		
	Control	at end	30 minutes later
Na penicillin	280	260	360
NaCl	180-260	200-220	160-200

There were no obvious differences in the electrocardiographic patterns which were recorded from needle electrodes inserted subcutaneously in the fore-legs. Both mice recovered fully and have continued to gain weight.

A fourth mouse received a total dose of 9,790,000 units per kg during 7 days (1,320,000-1,870,000 units per kg were injected

once daily intravenously every day but the second). Its weight dropped from 22.8 g to 19.1 g but returned to the pre-injection level 16 days after the last injection. It has since gained satisfactorily. The only other grossly apparent untoward sign was necrosis of one side of the tail probably owing to thrombosis at a site of injection. Control mice receiving saline intravenously recovered their pre-injection weights 6-10 days after the last injection and had no necrosis of the tail. Hematological changes were detected only in the mouse receiving the 6 repeated injections of Na penicillin. The total red-cell count fell to 4,300,000 (counts in control mice were 9,900,000 to 12,600,000) and was still below normal (6,800,000) 16 days after the last injection. A week later the number of erythrocytes per cmm was 9,700,000 and was regarded as normal. The leucocyte count varied considerably (12,000-27,000 per cmm); however, similar variations were encountered in control mice. On 2 out of 4 occasions (12 and 23 days after the last injection) there appeared to be an abnormal increase in the proportion of neutrophils (42-56%) at the expense of the lymphocytes which were reduced to 52-36%; the differential counts preceding each of these counts were less abnormal (26% neutrophils, 71% lymphocytes). There were 6-18% neutrophils and 81-93% lymphocytes in the blood smears of control mice.

Summary. Crystalline Na penicillin-G is not a hemolytic agent and is not adsorbed by rabbit erythrocytes suspended in isotonic saline solution. It does not alter the oxygen-consumption of yeast-cells, duck erythrocytes or slices of rat liver suspended in a solution containing 2.05 mg (3400 Oxford units) per ml. A high concentration (0.111M) causes a moderate reduction of the amplitude of each beat of the isolated frog's heart without affecting the rate; the effect is completely reversible. A concentration of 1-5500 in the bath fluid has no significant effect on the isolated ileum or uterus of the guinea pig and does not alter the response of the ileum to acetylcholine or of the uterus to posterior pituitary extract. A single intravenous dose as high as 3,800,000 units (2.32 g) per kilo is tolerated by the mouse as well as a corresponding volume of isotonic saline. One mouse received 6 intravenous injections in 7 days (total dose: 9,800,000 units or 5.93 g per kilo). During the first 2 weeks following injection there was a transient loss of weight and a transient anemia.

14658

Demonstration of the Properties A, B, M, N and Rh in Red-Cell Stromata.*

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The discovery of the Rh factor in human blood[1,2] and its role in erythroblastosis[3,4] suggests the possibility of preventing the disease by specific desensitization. As a starting material for the preparation of Rh hapten, large quantities of packed red cells are available from blood and plasma banks which have been established in most of the large hospitals.

The intact erythrocytes contain only relatively minute quantities of the group-specific substances, A, B, M, N, and Rh, so that the therapeutic injection of whole blood would undoubtedly be ineffectual unless large quantities were injected. Such a procedure would

* Aided by a grant from the United Hospital Fund of New York City.

1 Landsteiner, K., and Wiener, A. S., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 343.

2 Landsteiner, K., and Wiener, A. S., *J. Exp. Med.*, 1941, **74**, 309.

3 Levine, P., Katzin, E. M., and Burnham, L., *J. A. M. A.*, 1941, **116**, 825.

4 Levine, P., Burnham, L., Katzin, E. M., and Vogel, P., *Am. J. Obst. and Gyn.*, 1941, **42**, 925.

not be without danger, especially since the injection of large amounts of hemoglobin may cause dangerous renal complications. No method has yet been devised for extracting the group substances from erythrocytes in a purified form. However, by converting the red cells into stromata, the bulk can be considerably reduced and such red-cell stromata might find therapeutic application until methods are devised for the extraction of the individual blood properties in a pure form. The purpose of our study was to ascertain whether the process of converting intact red cells into stromata damaged the blood properties. If no damage occurred, one would expect an increase in the concentration of the group substances approximately equal to the reduction in volume resulting from the conversion of the red cells to stromata.

As a rule, the packed cells from a pint of blood were processed at one time; that is, we started with approximately 250 cc of cells. The cells were diluted to about 2 liters with saline solution and the suspension passed through a steam-driven Sharples Super-Centrifuge at approximately 200 cc per minute, with steam pressure of 10 lbs (approximately 10,000 rpm). The packed cells were resuspended in 2 liters of fresh saline and the process repeated. Where the washings did not appear almost colorless, the process would be repeated a third time. The packed cells were then diluted with 2 liters of distilled water, and after a detention period of 30 minutes the suspension was run through a Travis Colloid Mill. After this treatment the stromata were reduced to approximately uniform, minute particles, just visible under the high dry power of the microscope.[5] The particles were deposited by passing the suspension through the Sharples centrifuge, prepared with a cellophane liner, at a rate of 50 cc a minute, under steam pressure of 38 lb (50,000 rpm). The maximum yield was 10 cc of a semi-solid substance, which was light brown in color with a pink cast. With this technic the amount of group substance lost in the washings was apparently insignificant, as determined by quantitative inhibition tests on samples of the supernatants.

Tests were carried out to compare the concentration of group substances A and B in the original packed erythrocytes, red-cell stromata, and saliva of the corresponding group from secretors, by the inhibition or absorption method described by Wiener and Kosofsky.[6] Similar comparative tests were carried out on red cells and red-cell stromata for the properties M, N, and Rh.

The technic used in these experiments may be briefly described as follows: Serial dilutions (undiluted, $1:2^1$, $1:2^2$, $1:2^3$, etc) of packed red cells, red-cell stromata or saliva were prepared and one drop of each dilution of the material tested was placed in a series of test-tubes. A saline control was included in each series. A drop of a fixed dilution of the appropriate testing serum was added to each tube and the mixtures were allowed to react for one hour at room temperature. In the tests on the packed red cells, the supernatant fluids were transferred to another series of tubes at the end of the period of fixation, and a drop of the appropriate test cells was added to each tube. In the tests on stromata and saliva, the test cells were added directly to the original mixtures. One hour after the test cells had been added, the reactions were read, the absorption or inhibition titer being the last dilution of the material being tested which inhibited the agglutination of the test cells. For example, the first absorption titer in Table I is 2^6 or 64, indicating that this was the highest dilution of the packed red cells of group A_1 which completely absorbed the anti-A agglutinins in the group B testing serum. The reactions were usually very smooth, so that, for instance, in the case described above, dilution 2^7 gave a weak positive reading, while higher dilutions gave progressively stronger reactions, dilutions beyond 2^9 or 2^{10} giving as strong reactions as the saline control.

With regard to the testing sera, the group A and group B sera were produced in blood donors by isoimmunization and were selected

[5] *Cf.* Sigurdsson, B., *J. Exp. Med.*, 1943, **77**, 4.

[6] Wiener, A. S., and Kosofsky, I., *J. Immunol.*, 1941, **41**, 413.

TABLE I.
Comparison of Titers of Group Substances A, B, M, N, and Rh in Packed Red Cells and Red-Cell Stromata.

Group Substance	Material tested		Test Sera	Test Cells	Inhibition or Absorption Titer
A	Group A_1	cells	Group B	Group A_2	26
			" A	" B	0
		stromata	" B	" A_2	211
			" A	" B	0
		saliva	" B	" A_2	210
			" A	" B	0
B	Group B	cells	Group B	Group A_2	0
			" A	" B	25
		stromata	" B	" A_2	0
			" A	" B	28
		saliva	" B	" A_2	0
			" A	" B	29
M	Type M	cells	Anti M	Type M	20
		stromata	"	"	22
	Type MN	cells	"	"	21
		stromata	"	"	22
	Type N	stromata	"	"	0
N	Type N	cells	Anti-N	Type N	20
		stromata	"	"	21
	Type MN	cells	"	"	0
		stromata	"	"	0
	Type M	stromata	"	"	0
Rh_o	Type Rh_1	cells	Anti-Rh_o	Type Rh_1Rh_2	21
		stromata	"	"	25
	Type Rh_2	cells	"	"	20
		stromata	"	"	25
Rh′	Type Rh_1	cells	Anti-Rh′	Type Rh_1	20
		stromata	"	"	24
	Type Rh_2	cells	"	"	0
		stromata	"	"	0
Rh″	Type Rh_1	cells	Anti-Rh″	Type Rh_2	0
		stromata	"	"	0
	Type Rh_2	cells	"	"	20
		stromata	"	"	24

because of the ease with which the antibodies were neutralized in inhibition tests. The group A serum had a titer of 150 and was used in the tests in a dilution of 1:15; the group B serum had a titer of 600 and was used in a dilution of 1:20. The anti-M testing fluid had a titer of 15 and was used in a dilution of 1:3; the anti-N testing fluid had a titer of 8 and was used undiluted. All 3 varieties of anti-Rh sera used (anti-Rh_o, anti-Rh′, and anti-Rh″)† had titers of approximately 50 and were used in a dilution of 1:5. These were the same antisera which have already been described in detail in a previous publication.[7]

The results of a number of these experiments have been combined and summarized in Table I. It will be seen that the titers

† The designations of the Rh types and antisera correspond to the latest improved nomenclature proposed by Wiener (*Science*, 1944, **99**, 532).

[7] Wiener, A. S., Sonn, E. B., and Belkin, R. B., *J. Exp. Med.*, 1944, **79**, 235.

of the red-cell stromata for properties A, B, Rh_o, Rh′, and Rh″ are approximately 8 to 32 times as high as the corresponding titers of the original red cells. When one considers that 250 cc of red cells yield about 10 cc of red-cell stromata, the results are in satisfactfactory agreement with the expectations. In the case of the M and N properties, on the other hand, the expected rise in titer was not obtained, though there was in general a slight increase. This suggests that the processing damages the M and N properties.‡

It might be mentioned that the inhibition and absorption titers obtained for the various Rh properties have been consistently lower than the titers obtained for A and B. While it is true that it is not possible to compare the reactions of two antisera of different specificities, still these findings suggest that the number of Rh haptens per red cell may be less than the number of A, B, or O haptens. If this is proved to be correct, it might furnish an explanation for some of the peculiarities of the Rh *in vitro* reactions.

Red-cell stromata prepared with sterile precautions have been injected intramuscularly into human volunteers, without systemic or local reactions of any kind; also the intravenous injection of large quantities of stromata into rabbits appears to be innocuous, provided these are prepared with sterile precautions and smooth emulsions containing no coarse particles are injected. It would appear, therefore, that the clinical trial of the material for attempting the desensitization of Rh-negative mothers of erythroblastotic fetuses may be justified.

Summary. From 250 cc of packed erythrocytes approximately 10 cc of red-cell stromata can be obtained. The titers of the group substances, A, B, and Rh, in the stromata are almost proportionately higher than the corresponding titers of the original erythrocytes. However, the calculated rise in titer was not obtained for properties M and N, suggesting that these may have been damaged by the processing of the cells. The possible use of red-cell stromata for treating sensitized Rh-negative individuals is discussed.

‡ However, E. Witebsky and A. Heide (*Arch. Path.*, 1940, **30**, 1154) have reported the production of anti-N sera by injecting rabbits with boiled stromata of type N.

The authors wish to express their appreciation and indebtedness to Mr. Thomas Close of the laboratories of the Sharples Corp., Philadelphia, Penna., for his assistance and advice in the initial experiments on the preparation of red-cell stromata.

14659 P

Production and Implications of an Antiserum to Necrosin.*

VALY MENKIN.

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The writer has recently demonstrated that the pattern of injury in acute inflammation seems to be primarily referable to the liberation of a factor located in the euglobulin fraction of exudates.[1, 2] This factor has been termed necrosin. It is either a euglobulin or at least it seems to be associated with that fraction of exudates, *i.e.*, as obtained by precipitation with $(NH_4)_2SO_4$ at one third saturation. In addition, this fraction manifests pyrogenic and leukopenic properties.[2]

It occurred to the writer in connection with these studies that an antiserum to necrosin, if obtainable, may prove to be of theoretical interest and possibly also of clinical value. For this reason, in the fall of 1942, a series

* This is paper XXVIII of a series entitled "Studies on Inflammation."

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1 Menkin, V., *Science*, 1943, **97**, 165.

2 Menkin, V., *Arch. Path.*, 1943, **36**, 269.

of rabbits were immunized to varying doses of this fraction, in turn extracted usually from canine exudates by a technique previously described.[2] The immunizing procedure consisted of repeated subcutaneous injection of the material suspended either in physiological saline or in an aqueous medium. The injections were usually spaced at about 6 days apart. After about the fourth injection, a prominent edematous inflammation of the skin, with hemorrhagic necrosis, frequently developed. The reaction was far more intense than the effect of necrosin *per se*. The picture appeared to be typically that of an Arthus Phenomenon. From about a week to a month following the sixth injection of necrosin, blood was obtained either from the marginal vein of the ear or from the heart. The precipitin antibody titer was determined in the serum by utilizing necrosin as the antigen at various concentrations. Corresponding more or less with the elicited hypersensitive skin reaction (*i.e.*, Arthus Phenomenon), the antibody titer was found at high dilution of necrosin. A definite precipitin reading could be recorded at dilution of 1:100,000 of necrosin. On the other hand the interaction of normal rabbit serum with necrosin failed to induce any precipitin reaction.

These facts clearly indicate that necrosin or the euglobulin fraction of exudates is definitely antigenic, yielding as a result a serum containing a high antibody titer.‡ The implications of these findings are fairly obvious. For instance, it is conceivable that such a serum might be of value in hastening wound healing; in lessening the toxic or injurious manifestations of numerous inflammatory or infectious processes; and finally it may help in explaining the inability of the young animal to develop an adequate inflammatory reaction in contrast to the adult animal, as shown by Freund.[3] In other words, it is conceivable that the gradual development of resistance to infections occurring with age may in part be due to a subminimal immunization to necrosin, in turn liberated as a result of previous wounds or infections. These studies are being continued further by the writer.

Conclusions. Necrosin, the factor liberated in inflammatory exudates, and *per se* capable of explaining reasonably the basic mechanism of cellular injury in acute inflammation, is antigenic. As such, it is capable of inducing in the skin of sensitized rabbits a typical local anaphylaxis (Arthus Phenomenon), and in the serum of these same animals a high precipitin antibody titer. The implications of these observations are discussed.

‡ As indicated in the accompanying paper, No. 14660, entitled "Non-Antigenic Property of the Leukocytosis-Promoting Factor of Inflammatory Exudates," another protein fraction of canine exudate is *per se* essentially non-antigenic. Nevertheless, in an endeavor to determine the degree of specificity engendered by immunization with canine necrosin alone, similar observations are now in progress with the use of canine blood serum as the antigen.

[3] Freund, J., *J. Exp. Med.*, 1931, **54**, 171.

14660

Non-Antigenic Property of the Leukocytosis-Promoting Factor of Inflammatory Exudates.*

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Earlier studies have demonstrated the presence in inflammatory exudates of dogs, rabbits, and man of a factor responsible for the associated leukocytosis frequently encountered with inflammation.[1-3] These studies on rabbit exudates have been confirmed by Reifenstein and his collaborators.[4] Purification of exudative material has revealed that the active principle is either a pseudoglobulin or that it is at least associated with that particular fraction of exudates.[5-7] More recent studies have indicated that the leukocytosis-promoting factor (termed also LPF) not only causes a discharge of immature granulocytes from the bone marrow into the circulating blood stream; but that it likewise induces a hyperplasia of granulocytic elements as well as megakaryocytes in the marrow.[8] In view of the fact that the prognosis of many infectious processes is to a large extent referable to the number of circulating leukocytes,[9] it has been pointed out by the writer that this substance may prove to be of clinical significance in connection with a variety of inflammatory conditions as well as perhaps in certain hypoplastic states of the bone marrow.[8]

Before clinical usage of this active material is attempted, it is important to determine whether it is antigenic *per se*. The fact that it is protein-like in property, at least at its present stage of purification, does not necessarily indicate an antigenic capacity. As pointed out previously,[10] inflammation is a manifestation of severe cellular injury, and as such, the chemistry of the cell is profoundly altered. As a result of the basic cellular disturbance, various substances, wholly different in nature from ordinary known biochemical substances, are liberated in the exudate. These, in turn, are responsible for the fundamentally stereotyped reaction discerned in inflammation. Thus leukotaxine,[2] the leukocytosis-promoting factor, and necrosin[11] represent such active biological substances liberated by injured cells. As indicated in an accompanying communication,[11] necrosin, which seems to be linked to the euglobulin fraction of exudates, is antigenic in character. Is this state of affairs true also of the leukocytosis-promoting factor associated with the pseudoglobulin fraction of inflammatory exudates?

Several rabbits were repeatedly injected subcutaneously with samples of the leukocytosis-promoting factor. The injections were performed at intervals of about 6 days apart, for a period of about 4 to 5 weeks. In contrast to the effect of necrosin,[11] such injections induced no appreciable local cutaneous reaction as the period of immunization progressed; or at most an occasional rapidly transient acute inflammation developed which essentially disappeared in a day or two. There was no sustained skin manifestation

* This is paper XXIX of a series entitled "Studies on Inflammation."

† Aided by grants from the Johnson Research Foundation, New Brunswick, New Jersey, and by the Dazian Foundation for Medical Research.

1 Menkin, V., *Am. J. Path.*, 1940, **16**, 13.

2 Menkin, V., *Dynamics of Inflammation*, 1940, Macmillan Company, New York.

3 Menkin, V., Kadish, M. A., and Sommers, S. C., *Arch. Path.*, 1942, **33**, 188.

4 Reifenstein, G. H., Ferguson, J. H., and Weiskotten, H. G., *Am. J. Path.*, 1941, **17**, 233.

5 Menkin, V., *Arch. Path.*, 1940, **30**, 363.

6 Menkin, V., and Kadish, M. A., *Am. J. Med. Sci.*, 1943, **205**, 363.

7 Menkin, V., *Am. J. Med. Sci.*, 1944, in press.

8 Menkin, V., *Am. J. Path.*, 1943, **19**, 1021.

9 Robertson, D. H., and Fox, J. P., *J. Exp. Med.*, 1939, **69**, 229.

10 Menkin, V., *Arch. Path.*, 1943, **36**, 269.

11 Menkin, V., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 217.

which could truly be termed a classical Arthus Phenomenon or local anaphylaxis. Precipitin antibodies were also measured in the blood serum in an endeavor to determine whether the material, as in the case of necrosin, proved to be antigenic. In no instance was there any appreciable level of antibody found in the serum. The readings in the serological tubes were completely negative, or at most with high concentration of the antigen (*i.e.* the LPF) a mere trace of cloudiness could at times be discerned. The facts indicated that the leukocytosis-promoting factor obtained from canine exudates was essentially non-antigenic to the rabbit.

Subsequently, observations were made on guinea pigs with the leukocytosis-promoting factor of dog exudates in an attempt to determine whether the active material is capable of inducing a state of anaphylaxis. The leukocytosis-promoting factor contained in 1 cc of aqueous fluid was injected subcutaneously in several guinea pigs. About a month later a similar dose of the active material was reinjected intraperitoneally. In no case was there any evidence of the least sign of discomfort to respiration or any other anaphylactic manifestations. These observations corroborated further the evidence obtained in rabbits that the factor from exudates of another species is essentially non-antigenic. Furthermore, when the leukocytosis-promoting factor of human exudates was injected into a dog at varying intervals of about a month apart or thereabouts, there was no appreciable evidence found of any anaphylactic reaction following such injections.

Conclusions. The indications are that the leukocytosis-promoting factor of inflammatory exudates is essentially non-antigenic, *i.e.* when the active material derived from one animal form is introduced into an animal of a completely different species. The present observations should serve to dispel, in large part, any apprehension that may arise in regard to the possible clinical application of the leukocytosis-promoting factor.

14661

Upper Auriculo-Ventricular Rhythm (Coronary Sinus Rhythm) Experimentally Produced.*

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If an a-v rhythm presents inverted P-waves preceding the QRS-complex at a normal or slightly shortened distance, the focus of origin is supposed to be situated in that part of the a-v node which extends to the coronary sinus. This assumption is based on experiments of Zahn[1] who warmed this area by means of a thermode, and studies by Meek and Eyster[2] who used direct leads in order to locate the area of primary activity. Zahn did not record electrocardiograms, and some doubt was expressed to the effect that it is not possible to warm such a small focus (Meek and Eyster)[1] and that the determination of the precise site of stimulus formation in such small structures with the methods employed is "precarious" (Lewis).[3]

Therefore, it seemed desirable to obtain further evidence that stimulus formation takes place in the coronary sinus if the electrocardiogram described above is registered.

In a previous study it was demonstrated[4] that in hearts of dogs, perfused by the Langendorff method, warming of the coronary

* This investigation was aided by a grant from the United Hospitals Fund.

1 Zahn, A., *Arch. f. Physiol.*, 1913, **151**, 247.

2 Meek, W. J., and Eyster, S. A. E., *Heart*, 1914, **5**, 227.

3 Lewis, Th., *The Mechanism and the Graphic Registration of the Heart Beat*, 3rd ed., 1925.

4 Scherf, D., *Z. ges. exp. Med.*, 1931, **78**, 511.

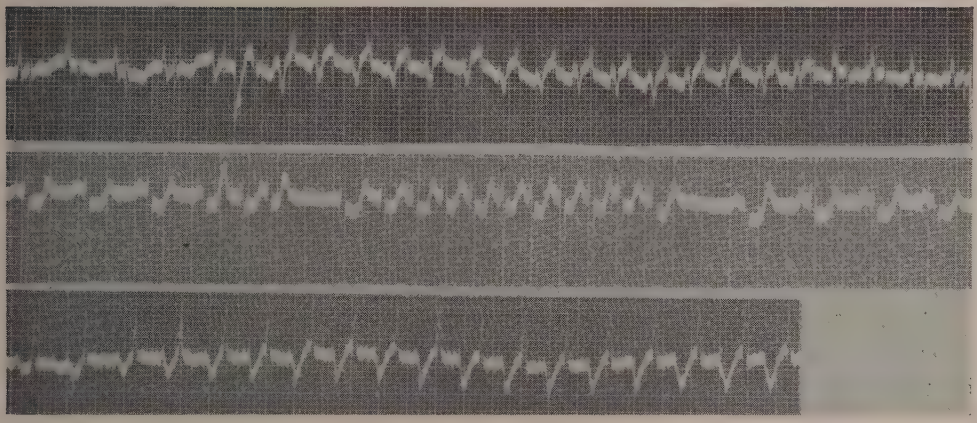

FIG. 1a.

Warming of the coronary sinus area through the wall of the coronary sinus vein causes coronary sinus rhythm with inverted P-waves and a normal P-R interval.

FIG. 1b.

Warming of the coronary sinus area directly, after opening the right auricle, causes a-v rhythm with simultaneous contractions of auricle and ventricle.

FIG. 1c.

Warming of the coronary sinus area through the coronary sinus vein induces coronary sinus rhythm with gradual increase of the P-R interval and deep negative P-waves.

TABLE I.

P-R Intervals During Sinus Rhythm and During Coronary Sinus Rhythm in 8 Experiments.

Date	P-R during sinus rhythm	P-R during coronary sinus rhythm
3-21-44	.11	.11
4- 4	.08	.08
18	.10	.10-.14
21	.08	.08
28	.08	.08-.10
5-20	.11	.11
2	.08	.08
16	.08	.08

sinus area after opening the auricle always evoked that form of a-v rhythm in which auricle and ventricle contract simultaneously. Since it was possible that in the Langendorff heart local disturbances of circulation prevent normal function of all parts of the a-v node, the experiments were repeated on the dog heart *in situ*.

The heart was exposed after artificial respiration was instituted. Nembutal anesthesia (0.5 cc per kg) was used. The electrocardiogram was registered in lead II. The area around the coronary sinus was warmed by a thermode which was applied through a small opening in the appendix of the right auricle (6 experiments). In order to locate the coronary sinus better, in 17 dogs the vena cava superior and inferior were clamped, the right auricle opened quickly, and the thermode applied to the orifice of the sinus vein especially on its medial aspect. In these experiments only the results obtained within 30 seconds after the clamping of the veins were used in order to eliminate influence due to disturbance of the nutrition of the a-v node. Control experiments showed, that clamping of both venæ cavæ for 50-60 seconds does not influence the length of the P-R interval.

In all experiments thus performed warming the coronary sinus area invariably caused an a-v rhythm in which the auricles and ventricles contracted simultaneously. No inverted P-waves were visible before the QRS-complex.

Different results were obtained however, when the heart was lifted slightly from the pericardial bed and the thermode was pressed on the terminal part of the coronary vein thus warming the area of the coronary sinus from behind. Eight experiments were performed in this way and each time the coronary sinus was repeatedly warmed through the thin wall of the coronary sinus vein or vena cava inferior. Without exception this caused a marked acceleration of the rate and the electrocardiogram showed inverted P-waves at a normal interval before the QRS-complexes.

Fig. 1a was obtained in a typical experiment. A sinus rhythm (rate: 166) exists at the beginning of the tracing. The P-waves are positive and rather high, the P-R interval measures 0.08 second and the QRS-complex shows a very thin, rather short S-wave due to the fact that the apex was slightly lifted from the pericardium. Warming of the coronary sinus through the coronary sinus vein causes an increase of rate up to 230. Deeply inverted P-waves appear and precede the QRS-complex by 0.08 second. The marked, positive Ta-wave moves the ventricular complex slightly above the zero line. The thermode was immediately removed after the cardiac acceleration occurred and the original sinus rhythm is quickly restored.

Fig. 1b shows in the beginning a regular sinus rhythm which was registered after opening the right auricle. Warming of the orifice of the coronary sinus vein induced a short period of tachycardia during which no P-waves were visible because auricle and ventricle contracted simultaneously.

Fig. 1c shows the gradual change from a sinus rhythm to an accelerated coronary sinus rhythm which appeared during warming of the coronary sinus area through the wall of the coronary sinus vein. Due to the tachycardia the P-R interval increases from 0.13 to 0.15 second.

Table 1 shows the P-R intervals during the sinus rhythm and during the coronary sinus rhythm in 8 experiments. In all experiments the P-R distance remained unchanged or became slightly larger during the a-v rhythm. Lengthening appeared at the height of the tachycardia if the thermode was kept in place for a longer time and may be attributed to a fatigue of the conduction to the ventricle.

The experiments prove that stimulus formation in the dog heart, originating in the area around the coronary sinus, is accompanied by inverted P-waves followed by QRS-complexes at a normal interval.

According to Kung[5] the prolongation of the a-v node to the coronary sinus area joins the node on its posterior aspect. It contains many intermuscular ganglion cells. This position of this extension may explain why the warming of area around the mouth of the coronary sinus vein from the endocardial side did not elicit coronary sinus rhythm.

Conclusion. Warming of the area of the coronary sinus in the dog heart *in situ*, after opening of the right auricle does not cause coronary sinus rhythm.

This rhythm may be readily elicited if the coronary extension of the a-v node is warmed through the wall of coronary vein.

[5] Kung, *Arch. f. exp. Path. and Pharm.*, 1930, **155**, 295.

14662

Effect *in vitro* of Curare Alkaloids and Crude Curare Preparations on "True" and Pseudo-Cholinesterase Activity.

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In previous publications,[1] we reported that intocostrin, which is prepared from an Indian trade curare, strongly inhibited the choline esterase activity of human serum. It was pointed out that this preparation of curare was not a pure chemical substance and that it would be desirable to investigate some of the pure curare alkaloids. Since it has been found that crude curare preparations vary in their lissive action and also in toxicity further information regarding the constituents of the crude preparations should be of value in clinical and physiological studies.

Recently McIntyre and King[2] reported that d-tubocurarine chloride had no inhibiting effect on the choline esterase activity of serum although it produced muscular paralysis. We were fortunate in having placed at our disposal for this study the following pure curare alkaloids; d-chondocurine, d-isochondodendrine, d-isochondodendrine dimethyl-ether, alkaloid 4, and d-tubocurarine chloride which had been isolated from chondodendron tomentosum.*

It had been shown by a number of investigators[4, 5] that the red blood cells of human blood contain a cholinesterase (called "True cholinesterase") which differs from that present in serum (called pseudo-cholinesterase). The effect of the alkaloids upon both enzymes therefore was investigated using acetylcholine bromide and mecholyl (acetyl-beta-methyl-choline) as substrates. (Note: The "pseudo-esterase of serum will not hydrolyze mecholyl whereas the "true" esterase hydrolyzes both acetylcholine bromide and mecholyl; the latter substrate is therefore specific for the "true" esterase.[4] Benzoylcholine, which is specific for the pseudo-esterase, was not available for study).

Procedure. The alkaloid solutions were prepared as follows: d-tubocurarine chloride was dissolved in distilled water; d-isochondodendrine and alkaloid 4 were dissolved in a few milliliters of N/100 HCl and d-chondocurine in N/100 H_2SO_4 and made up to volume with distilled water so that one milliliter was equivalent to approximately one milligram of alkaloid.

The enzymatic hydrolysis was determined by the continuous electrometric titration method[5] at pH 7.35 and 37°C; 0.2 ml of serum or 0.2 ml of washed hemolyzed red blood cells of human blood prepared according to Mendel,[4] was used in a total volume of 20 ml. The solution contained approximately 0.7% of sodium chloride in order to minimize the error due to varying concentrations of sodium. A 0.2% solution of acetylcholine iodide or bromide or mecholyl was used as substrate. N/100 NaOH was used to neutralize the acetic acid liberated by the enzymatic hydrolyses. Burette readings taken every 5

* Dr. O. Wintersteiner and Dr. J. D. Dutcher of the Squibb Institute for Medical Research recently isolated these compounds and kindly supplied them for this study. Chemical and physiological data regarding these compounds are given in their paper.[3] They have also supplied us with various other crude curare preparations which have been included in this study as indicated in the table. According to a communication from Dr. Wintersteiner, only *d*-tubocurarine, which is a quaternary base, possesses the typical curare (lissive) action. The other alkaloids mentioned are tertiary bases and were found ineffective in the test for lissive action.

1 Harris, M. M., and Harris, R. S., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 619; *Ibid.*, **46**, 623.

2 McIntyre, A. R., and King, R. E., *Science*, 1943, **97**, 69.

3 Wintersteiner, O., and Dutcher, J. D., *Science*, 1943, **97**, 467.

4 Mendel, B., and Rudney H., *Biochem. J.*, 1943, **37**, 59; *Ibid.*, **37**, 473.

5 Alles, G. A., and Hawes, R. C., *J. Biol. Chem.*, 1940, **133**, 375.

TABLE I.*

Effect of Various Curare Alkaloids on the Cholinesterase Activity of Serum and Hemolyzed Red Blood Cells at pH 7.35 and 37°C.

Alkaloid		Cholinesterase activity					
		Acetylcholine bromide substrate				Mecholyl substrate	
		Serum		Hemolyzed R.B.C.		Hemolyzed R.B.C.	
Type	Amount mg	Units	% inhibition	Units	% inhibition	Units	% inhibition
No. 1	0	1.18	0	1.86	0	1.30	0
d-chondocurine	0.5	.88	25	1.25	33	.94	27
	1.0	.76	36	.99	47	.81	37
No. 2	0	1.27	0	1.95	0	1.34	0
d-isochondodendrine	.25	1.13	11	1.93	1	1.29	4
	.50	1.05	17	—	—	1.23	8
No. 3	0	1.11	0	2.17	0	1.61	0
Alkaloid 4	.53	.45	59	1.13	48	.77	52
No. 4	0	1.03	0	2.16	0	1.45	0
d-Tubocurarine chloride	1.04	.89	14	—	—	—	—
	2.0	.69	33	1.82	16	1.11	24
No. 5	0	1.15	0	2.23	0	1.57	0
d-isochondodendrine dimethylether	1.0	.66	43	1.93	13	1.24	20
No. 6	0	1.11	0	2.17	0	1.67	0
Intocostrin (Lot No. 69904) (unauthenticated curare)	0.016	.13	88	2.11	3	1.49	11
No. 7	0	.99	0	2.09	0	1.36	0
Curare 4901-1″ (unauthenticated)	0.01	.06	94	2.02	3.5	1.40	—1.7
No. 8	0	1.30	0	2.27	0	1.56	0
Curare (Gill) (Lot No. 25064)	.25	.91	30	2.10	4	1.47	6
No. 9	0	1.26	0	2.34	0	1.58	0
Serpa A	.25	.83	34	2.19	6	1.53	3
No. 10	0	1.19	0	2.76	0	1.79	0
Curare (Gill), ether extract†	.05	.66	44	2.59	6	1.75	2
No. 11	0	1.27	0	2.76	0	1.91	0
Curare (Gill), chloroform extract†	.1	.87	31	2.59	6	1.76	8

* This table contains only representative data of many experiments carried out with the various alkaloids.

† The chloroform and ether extracts from curare (Gill) contain, in varying proportions, the tertiary alkaloids listed in the table and perhaps others which have not been isolated.

minutes for 20 minutes and plotted against time gave a straight line. The amount of acetic acid liberated in 20 minutes by the choline esterase activity of 0.2 ml of serum, or hemolyzed red cell solution expressed in terms of the milliliters of N/100 sodium hydroxide required for its neutralization, was used as the measure of the enzymatic activity and one milliliter is called one unit of esterase activity.

The effect on the choline esterase activity of the addition of varying amounts of the different alkaloids was determined. Corrections were made for the spontaneous hydrolysis of the acetylcholine derivatives. Preliminary tests showed that all of the alkaloids remained in solution at pH 7.35. However, at a more alkaline pH some of the pure alkaloids tended to precipitate.

Observations. (a) *Effect of Alkaloids on "Pseudo-cholinesterase" of Serum.* It will be seen from the table that d-tubocurarine

chloride had the least inhibiting effect on the enzymatic hydrolysis by pseudo-cholinesterase of any of the pure or crude alkaloid preparations tested. In comparison d-isochondodendrine was approximately twice as effective, d-chondocurine three times, and alkaloid 4 about 8 to 10 times as effective as d-tubocurarine chloride. Intocostrin (No. 6) one of the preparations used in the studies previously reported[1] which was prepared from unauthenticated curare was about 1500 times as effective while intocostrin prepared from curare (Gill) had an inhibiting effect slightly less than that of d-isochondodendrine. (1 mg gave 21% inhibition, data not given in the table).

Preparation (No. 7), some of the unauthenticated curare which originally had been used to prepare intocostrin, supplied to us by Dr. Wintersteiner, also had a similar marked inhibiting effect. All of the other curare preparations (No. 8, 9, 10, 11) had an exceedingly weaker inhibiting effect.

(b) *Effect of Alkaloids on "True Cholinesterase."* The unauthenticated curare (No. 6 and 7) in the amounts which almost completely inhibited the pseudo-esterase had little or no effect upon the true esterase. The other curare preparations (No. 8, 9, 10, 11) also had only a slight inhibiting effect on the latter enzyme in contrast to the effect upon the pseudo-esterase. This is in contrast to the effect produced by the pure alkaloids (especially No. 1 and No. 3) where both esterases are equally affected.

Discussion. It will be noted that, contrary to the report of McIntyre and King,[2] d-tubocurarine chloride has some inhibiting effect on choline esterase activity of both the true and "pseudo-cholinesterase" *in vitro*. However, this is not demonstrable in the low concentration of the alkaloid. It is possible that a difference in methods from those used by the above investigators may account for the difference between their findings and ours.

While the tertiary alkaloids which were tested do not possess the lissive action which is manifested by the quaternary alkaloid, d-tubocurarine chloride, they have a more pronounced inhibiting effect upon the cholinesterases. However, it would not be safe at present to conclude that the lissive action is therefore totally unrelated to the effect on the cholinesterases.[6,7]

The findings with the different curare preparations is of considerable interest. The marked inhibition of the pseudo esterase by the unauthenticated curare with little or no effect upon the true esterase is further evidence for the existence of 2 enzymes. It will be of interest to determine the nature of the substance producing this specific effect upon the pseudo-esterase. It would offer a means of studying the effects of inhibiting this esterase while the true esterase remains active. Information regarding the physiological role of the pseudo esterase may thus be obtained.

The inhibiting effect of the curare specimens (No. 8, 9, 10, and 11) upon the pseudo esterase, although considerably less than that of the unauthenticated curare, cannot be accounted for by the pure alkaloids investigated and points to the presence of some other inhibiting substance. Whether this is due to a small amount of the same substance as that in the unauthenticated curare cannot be stated at present. The marked inhibition reported in our previous studies is now shown to have been due to the effect upon the pseudo-esterase of some substance in the crude curare preparations.

Summary. (1) Five pure curare alkaloids and several curare preparations were investigated for the effect on the activity of "true" and pseudo-cholinesterase. (2) A very active substance which specifically inhibits the pseudo-esterase has been found in some curare preparations. (3) The value of this substance for special studies regarding the role of the pseudo-esterase is discussed. (4) The difference in the inhibiting action of the pure alkaloids and the crude curare preparation is demonstrated.

[6] Roepke, M. H., *J. Pharm. and Exp. Therap.*, 1936, **59**, 264.

[7] Mendel, B., and Hawkins, R. D., *J. Neurophysiol.*, 1943, **6**, 431.

14663

Effect of Parenterally Administered Citrate on the Renal Excretion of Calcium.*

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There is an increasing body of evidence for the importance of citric acid in the metabolism of calcium. Shohl and Butler[1] found that in the treatment of infantile rickets citrate has a vitamin D-like action. Dickens[2] reported that bone contains as much as 1.5% citric acid, and that the citrate content of bone is increased by the administration of parathormone. Letonoff and Kety[3] showed that citric acid mobilizes lead from the bones. Scott, Huggins and Selman[4] confirmed previous findings that the renal excretion of citrate is decreased in patients suffering from urolithiasis.

In the experiments to be reported here the effect of the parenteral administration of sodium citrate on the renal excretion of calcium and phosphorus and on the histology of bones was studied.

Experimental. The animals used were 7 adult dogs, weighing 10 to 15 kg, 4 puppies and 6 rats.

1. The adult dogs had cannula-cystostomies performed upon them about 1 week before the experiment. They were fasted for 17 hours. On the morning of the experiment they were fastened to a device essentially similar to that described by Huggins and Clark[5] and kept standing on their feet during the periods of urine collection. At 9:00 a. m. they were given about 200 cc of water by stomach tube. Flasks were attached to and removed from the cannula at 30 minute intervals from 9:30 on; specimens were collected at 10:00, 10:30, 11:00, 11:30, and finally one for the interval from 4:00 to 4:30 p. m. At 10:30 100 cc of a 4% solution of sodium citrate was injected subcutaneously. No reactions of any kind were observed. Blood samples were taken at 9:30 and 11:30 a. m. and at 4:30 p. m. Calcium was determined by the Clark-Collip[6] modification of the Kramer-Tisdall[7] method; inorganic phosphorus by the photoelectric elon method.[8] The urine samples were ashed.

Table I shows the results in 4 of the animals. In the remaining 3 animals the results were essentially similar. Urinary calcium and phosphorus are given in 30-minute totals.

As shown in the table, the injection of citrate is followed by a rapid increase in the rate of renal elimination of calcium. A high level of excretion is reached after 60 minutes which later declines, and 5 hours after the injection it is practically back to its initial level. The excretion of phosphate does not follow any typical course, except for a tendency to a late increase in excretion. The blood levels of both calcium and phosphate remain essentially unchanged throughout. The marked increase in the renal clearance of calcium is obviously due to the fact that a larger fraction of the plasma calcium becomes ultrafiltrable.

2. Two puppies (A and B), weighing 1500 and 3000 g, respectively, were put in individual metabolism cages and fed a standard diet. For a control period of 2 days

* This work has been done under a grant from the Douglas Smith Foundation for Medical Research of the University of Chicago.

1 Shohl, A. T., and Butler, A. M., *New England J. M.*, 1939, **320**, 515.

2 Dickens, F., *Bioch. J.*, 1941, **35**, 1011.

3 Letonoff, T. V., and Kety, S. S., *J. Pharm. and Exp. Therap.*, 1943, **77**, 151.

4 Scott, W. W., Huggins, C., and Selman, B. C., *J. Urol.*, 1943, **50**, 202.

5 Huggins, C., and Clark, P. J., *J. Exp. Med.*, 1940, **72**, 747.

6 Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, 1925, **63**, 461.

7 Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, **47**, 475.

8 Gomori, G., *J. Lab. and Clin. Med.*, 1942, **27**, 955.

TABLE I.
Effect of Citrate on Urinary Excretion of Ca and P.

Time	4% citrate inj.	Urinary Ca 30 min. (mg)	Urinary P 30 min. (mg)	Serum Ca mg %	Serum P mg %	4% citrate inj.	Urinary Ca 30 min. (mg)	Urinary P 30 min. (mg)	Serum Ca mg %	Serum P mg %
	Dog 509					Dog 602				
9:30 AM		—	—	11	3.9		—	—	10	5.5
10:00		0.36	1.96				0.52	0.54		
10:30	100 cc	0.31	0.44			100 cc	0.57	0.85		
11:00		0.64	0.30				1.04	0.83		
11:30		9.0	9.1	11	3.7		1.78	0.83	10	5.5
4:30 P.M.		0.59	15.5	11	3.8		0.42	2.0	9	6.4
	Dog 213					Dog 188				
9:30 AM		—	—	10	3.7		—	—	10	5.1
10:00		0.70	2.87				0.49	3.3		
10:30	100 cc	0.77	0.39			100 cc	0.34	6.9		
11:00		3.7	0.42				1.4	8.3		
11:30		10.0	7.1	10	3.1		7.8	7.8	12	4.0
4:30 PM		0.55	1.86	11	4.6		0.36	7.3	9	3.9

TABLE II.
Urinary Excretion of Ca and P in 2 Puppies Under Control Conditions and Under Citrate Treatment.

		Urinary excretion (mg/24 hours)			
		Ca		P	
Day	Treatment	Puppy A	Puppy B	Puppy A	Puppy B
1	None	0.36	15	23	110
2	None	0.88	31	47	120
3	30 cc saline/kg	0.71	25	86	104
4	30 " " "	2.0	15	78	84
5	30 " citrate/kg	20	75	90	174
6	30 " " "	30	64	73	155

they were left untreated; for the following 2 days they received 30 cc/kg of normal saline subcutaneously, divided into a morning and an evening dose; for the last 2 days they were given 30 cc/kg of 4% sodium citrate in the same way. The daily amounts of urine were analyzed for calcium and phosphorus.

Table II shows the results.

It is clearly visible that on the citrate days the excretion of calcium was considerably increased.

Puppy B was found dead in its cage on the morning of the eighth day; autopsy revealed pulmonary edema and some edema of the subcutaneous tissues throughout the body. The microscopic changes in the bones will be described under experiment 3.

3. Puppy C, weighing 1300 g, was given 2 daily injections of 7 cc of 4% sodium citrate each; Puppy D, weighing 1500 g, was given 2 daily injections of 15 cc each. On the afternoon of the third day biopsies of the tibial crests were performed. Microscopic examination of the specimens showed hyperemia

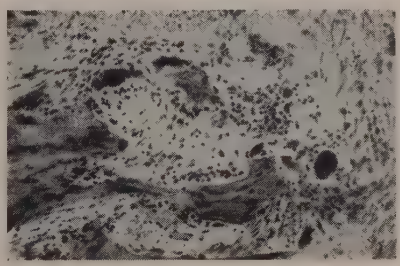

FIG. 1.
Osteoclastic absorption of bone and fibrosis of bone marrow in the upper tibial metaphysis of Puppy B.

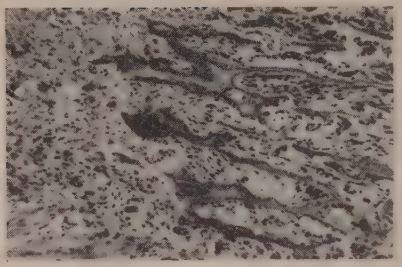

FIG. 2.
Similar changes in the rib of a citrate-treated rat, close to the costochondral junction.

and edema of the bone marrow, transformation of osteoblasts into spindle cells, an increase in the numbers of osteoclasts far beyond the limits of normal, fragmentation of bone trabeculae, and areas of early fibrosis—a picture extremely similar to that seen after toxic doses of parathormone. Very similar but even more severe changes were seen in the metaphysis of the tibia and in the costochondral junctions of Puppy B.

4. Three rats, weighing 100 g each, and 3 others, weighing 50 g each, were given subcutaneously a daily amount of 60 cc/kg of a 4% sodium citrate solution, divided in 2 doses, for 5 days. After 5 days the animals were killed and their bones examined microscopically. The bones of the heavier animals did not show clear-cut changes, while the bones, especially the ribs, of the smaller animals presented lesions essentially identical with those seen in the puppies, although the changes in the rats were much milder.

Summary. The subcutaneous injection of 8 to 30 cc/kg of a 4% solution of sodium citrate into dogs causes a considerable and prompt increase in the urinary excretion of calcium while leaving the blood calcium level essentially unaffected. Repeated subcutaneous injections of citrate into puppies and young rats lead to osseous changes very similar to those produced by large doses of parathormone.

14664 P

Management of Acute Myocardial Infarction by Narcosis.

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The relief of pain, anguish, and apprehension is a very important objective in the management of the initial phase of acute coronary occlusion and myocardial infarction. Morphine and barbiturates are commonly relied upon to secure this, but fail to do so in a satisfactory manner in a number of patients. Even after the abatement of pain, the exhibition of extreme restlessness with much tossing about after repeated administration of these drugs in therapeutic doses indicate the pressing need for some departure in the present almost standardized scheme of therapy. The frequent persistence of a considerable degree of excitement is perhaps more damaging than pain in raising the metabolic activity of the tissues and in imposing an additional burden on an already injured heart at a most critical period. It is therefore proposed on theoretical grounds, that the induction of a sufficiently deep narcosis for an arbitrary period of several days is worthy of trial in selected cases of this disease, notwithstanding certain hazards likely to be entailed by the procedure. By its adoption, however, the prime therapeutic desideratum, namely, that of reducing body metabolic activity, is immediately accomplished, and maintained over the period of narcosis. Three factors operating in this direction are: (1) the relaxation of the body musculature; (2) the almost total deprivation of food; and (3) the abolition of pain and apprehension. It is conceivable that during the prolonged period of sleep, there

would be less likelihood of the production of reflex vasoconstriction in other portions of the coronary tree, and that healing processes might be initiated earlier, since the burden on the heart is lessened.

The following case record is that of a very apprehensive male negro 31 years old, weighing 185 lb., who was admitted 7 hours after an attack diagnosed, and later confirmed, as a recent myocardial infarction. There was a history of chronic alcoholism, hypertension, and syphilis. Forty grains of sodium amytal were given intravenously in divided doses over a 72-hour period. Morphine gr. 1/6 and atropine gr. 1/150 were given hypodermically every 4 hours. He was placed under an oxygen tent with continuous nursing care. A state of narcosis was induced for 71 hours of an observation period lasting 84 hours. The periods of deep sleep or narcosis had a duration of between 4 and 8 hours. There were 11 interruptions in the 84-hour period, occasioned by the hourly inhalations of CO_2 to prevent atelectasis and pneumonia. During these interruptions he manifested considerable thirst and hunger and voided frequently. Urinary incontinence occurred one time; there was no bowel movement. He received 300 cc of fluids daily by subcutaneous or oral route, but chiefly by the former, and this included 1000 cc of normal saline. His food consisted of milk and eggs to total 1600 calories.

During the short periods of wakefulness, he manifested marked drowsiness, but was coherent. On two occasions, he complained of mild precordial pain, and another time, of itching. Restlessness did not reappear. At the conclusion of the period, he was given small doses of barbiturates by mouth, which have been continued to the 16th day after infarction. The patient's convalescence has been uneventful, and his recollection of the early management very pleasant.

Summary. The induction of continuous deep sleep or narcosis by the use of barbiturates given intravenously is suggested for the management of the early stages of acute coronary occlusion and myocardial infarction. This was tried rather successfully on one patient. The inability to produce narcosis of the depth originally planned was probably prevented by the chronic alcoholism.

14665 P

Tissue Culture Studies of Cytoplasmic Inclusion Bodies in Lymph Nodes of Hodgkin's Disease.*

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In the literature, the etiology of Hodgkin's disease has been ascribed to numerous agencies. Among these, bacterial and virus infections have been included.

This report is based on nodes from 35 cases of Hodgkin's disease, obtained surgically from Memorial Hospital, New York. The lymph nodes were from early and late stages of the disease. As controls, normal lymph nodes from 12 radical mastectomy cases were used, also nodes from various lymphomas (20 lymphosarcomas, 15 leukemias) and from 23 metastatic carcinomas and 10 lymphadenitis cases.

The nodes were cut into small fragments (explants, about 1 cu mm in size) from which tissue cultures were prepared in the usual way. The medium consisted of a mixture of fowl plasma, human serum and chick embryo extract. In some cases the serum was from patients with advanced stages of Hodgkin's disease.

The cultures were maintained for a period varying from a few days to several weeks.

* This investigation was aided by the International Cancer Research Foundation, Philadelphia.

After 24 hours' incubation, granulocytes, eosinophiles, and lymphocytes had migrated from the explant. After 48 hours the outgrowth contained also macrophages, reticulocytes, and fibrocytes. After 48 to 72 hours of incubation there also appeared, on the periphery of the explants, large multinucleated giant cells with oval nuclei. The nuclei tended to surround a relatively large, grayish and granular region generally occupying the central portion of the cell. These cells, identified as Reed-Sternberg cells, were found in every case of true Hodgkin's disease and were absent in the other lymphomas and in the normal lymph nodes. The longer the tissue cultures were maintained, the more numerous and larger were these cells.

Brilliant cresyl blue (1:50,000), used as a vital dye for virus cell inclusions, stained the granules of the central body of the Reed-Sternberg cell within 15 minutes after exposure of the culture to the dye. The stained granules were irregular in shape and size. Many appeared to be clumps of smaller granules. The color varied from red to purple of different intensities. The granules of the central body did not stain with Janus B green (1:20,000). Fibrocytes, macrophages, and lymphocytes in the same cultures regularly showed cytoplasmic inclusions within vacuoles, varying in shape and size, which gave the same reddish staining reaction with brilliant cresyl blue as the granules in the central body of the Reed-Sternberg cell. However, cells of the same types obtained from control lymph nodes and grown in a normal medium contained no granules giving the specific coloration with brilliant cresyl blue.

Non-cellular extract of nodes of Hodgkin's disease was obtained from the supernatant fluid of tissue cultures of nodes grown for 14 days and centrifuged at 2000 r.p.m. for half an hour. Fragments of normal lymph nodes were grown for 6 days in a culture medium containing this extract. When vitally stained with brilliant cresyl blue, the lymphocytes and macrophages of these nodes showed cell inclusions within vacuoles of the type found in cultures of nodes of Hodgkin's disease. There was no evidence of giant cells resembling the Reed-Sternberg cells. Control cultures exposed to normal medium did not show the brilliant cresyl blue inclusions.

Supernatant extract from the Hodgkin's disease cultures was injected on to the surface of the chorio-allantoic membrane of 23 hens' eggs which had been incubated for 6 to 11 days. These eggs were incubated for 6 more days and then examined. Eight eggs were found to have vesicles in grape-like clusters measuring about 3 mm in size. The vesicles contained a clear fluid. As controls, 10 eggs were injected with fluid from normal lymph node cultures and 11 eggs with fluid from lympho-sarcoma cultures. None of these showed any lesions.

Portions of the chorio-allantoic membranes containing the lesions produced by the Hodgkin's node extract were grown in tissue cultures for 48 hours and then vitally stained with brilliant cresyl blue. Many of the cells in these cultures showed the specific cell inclusions found in the original tissue cultures of Hodgkin's disease. These inclusions were never found in cultures of chorio-allantoic membranes inoculated with extracts of normal lymph-nodes or of lympho-sarcomas.

Tissue cultures of all the experiments were fixed every 2 or 3 days, and stained *in toto* with various virus inclusion stains. Of these, Seller's was found to be the best. With this stain only the cell inclusions which color specifically with brilliant cresyl blue took on the red color of the basic fuchsin, while methylene blue colored the nuclei and the usual cytoplasmic granules as well. With the hematoxylin-eosin method, the central zone of the Reed-Sternberg cell proved to be highly basophilic.

Preparations were also stained with Ziehl-Neelson's and Gram's bacterial stains. No bacteria could be demonstrated. As is well known, the tissue culture medium is favorable for the growth of aerobic bacteria. In all the cases of Hodgkin's disease, involving many hundreds of cultures, no bacterial, fungal or protozoan contamination was ever observed.

14666

Evaluation of a Satisfactory Operation for Ulcer.

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From the University of Minnesota.

For half a century surgeons have been performing operations upon the stomach for the relief of ulcer without knowing actually what are the criteria of a successful operation. A few years ago, an effort was made in this clinic to attempt to define some of the attributes of a satisfactory operation for ulcer. Within a few years,[2] it became apparent what some of the characteristics of a satisfactory operation are for ulcer in man. In the meanwhile, employing Code's scheme of liberating histamine slowly from beeswax,[3] a satisfactory scheme for producing ulcer in experimental animals has been developed.[4] By implanting daily a suitable dose of histamine-in-beeswax, ulcer can be produced quite regularly in the majority of commonly available small animals.[5] This method of producing ulcer affords an excellent scheme of assessing the protection afforded by a given operation. It is the purpose of this communication to report the observations and experiences of such a study.

Procedure. Adult mongrel dogs only were used in this study. Each procedure (Fig. 1) was performed in 4 dogs. All anastomoses were done by employing the closed aseptic technique. Following operation, the dogs were allowed to recover for 3 months. They were then injected daily with 30 mg of the histamine-beeswax mixture; the injections being made intramuscularly along the spinal muscles and multiple sites were used to permit a better distribution and liberation of the histamine. The injections were done at about 5:00 p.m. following which the dogs' feed pans were removed and no more food given until the following morning. All the surviving animals were sacrificed at the end of 40 days. Obviously moribund animals were sacrificed earlier in order to obtain fresh specimens. Control dogs were injected with each series of animals.

Results. Group I. Gastrojejunostomy—Two of the dogs died after 3 and 13 injections respectively, of large 1.5 to 2 cm perforated jejunal ulcers. The other 2 dogs survived the injection period and at autopsy both showed large jejunal ulcers. All ulcers were on the jejunal side of the anastomosis and there were no duodenal or gastric ulcers.

Group II. Small Gastric Resection or Antral Excision—All dogs developed large jejunal ulcers from 1 to 3 cm in diameter. Three of the 4 dogs survived the 40-day injection period. The other dog was sacrificed after 35 injections; moribund from hemorrhage from a large jejunal ulcer.

It is thus apparent that neither of these two procedures furnished any protection against the development of ulcer.

Group III. Extensive Gastric Resection, 75%, and Group IVA, Extensive Gastric Resection with Excision of Antral Mucosa will be considered together as they are fundamentally the same operation except for a technical variation in inversion of the duodenum.

In the IVA operation, the antral mucosa is also excised, and the antral musculature is utilized to effect a satisfactory closure of the duodenal stump. None of the 8 dogs in these two groups developed any evidences of jejunal ulcer and all dogs survived the 40-day

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1 Wangensteen, O. H., Varco, R. L., Hay, L., Walpole, S., and Trach, B., *Ann. Surg.*, 1940, **112**, 626.

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4 Walpole, S. H., Varco, R. L., Code, C. F., and Wangensteen, O. H., Proc. Soc. Exp. Biol. and Med., 1940, **44**, 619.

5 Hay, L. J., Varco, R. L., Code, C. F., and Wangensteen, O. H., *Surg. Gynec. and Obst.*, 1942, **75**, 170.

6 Edkins, J. S., *J. Physiol.*, 1906, **34**, 133.

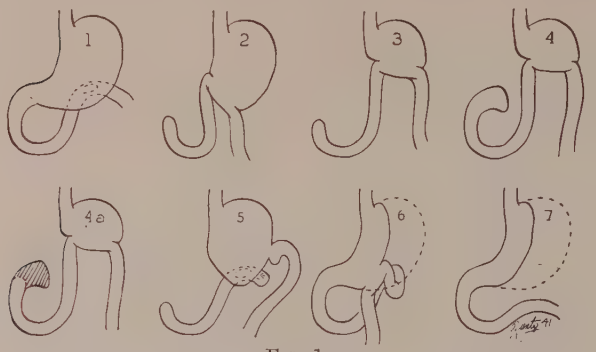

FIG. 1.

Types of operative procedure employed in assessing its protective influence against the development of ulcer provoked by histamine-in-beeswax:

1. Gastrojejunostomy.
2. Antral excision.
3. Three-quarter gastric resection.
4. Finsterer exclusion operation.
4A. Finsterer exclusion operation with removal of antral mucosa (size of gastric resection the same in 3, 4, and 4A).
5. Schmilinsky operation of antral excision with total intragastric regurgitation.
6. Fundusectomy with gastrojejunostomy.
7. Fundusectomy.

injection period. In 4 of the animals a few scattered superficial erosions were noted in the gastric pouch but the jejunum in each instance was entirely normal. This operation would thus appear to furnish satisfactory protection against the development of ulcer.

Group IV. Finsterer Antral Exclusion—Three of the 4 dogs in this series survived the 40-day injection period. There were no evidences of jejunal ulceration or erosion; however, numerous submucosal hemorrhages and superficial erosions were noted throughout the gastric pouches.

The other dog expired after 32 injections and showed a rather marked jejunitis and gastritis of the residual pouch but no definite ulcer.

This operation as well as the small gastric resection (Group III) offer the opportunity of testing the validity of the Edkins' "gastric" hypothesis. The antrum is alleged to be the source of a hormone "gastrin" which stimulates the secretion of acid from the corpus and fundus of the stomach.

The results noted in the dogs upon which the small gastric resection was done would tend to invalidate this thesis, for despite complete excision of the antrum, all animals developed jejunal ulcer. There are more changes noted in the gastric pouch in the antral exclusion operation but the findings in the jejunum are not unlike those in which the antrum has been removed. It would thus appear that this operation would not support the Edkins' thesis either, although the changes in the gastric pouch might be interpreted as evidences of increased stimulation.

Group V. Schmilinsky Operation. This procedure includes antral excision with implantation of the proximal loop on the fundus of the stomach to insure complete intragastric regurgitation. Three of the 4 dogs died after 2, 8, and 11 injections, respectively, of large perforated jejunal ulcers varying in size from 1 to 3 cm. The other dog survived the injection period but showed a typical jejunal ulcer at autopsy. It is thus apparent that this operation increases the development of jejunal ulcer rather than offering any protection.

Group VI. Fundusectomy with Gastrojejunostomy. All dogs survived the injection

period. One dog developed a superficial duodenal erosion and 2 dogs developed a rather marked antral gastritis. No definite pathologic change was noted in the other dog.

Group VII. Fundusectomy. This procedure includes excision of the acid-secreting area, as described by Connell. All dogs survived the injection period. One dog developed a 1 cm duodenal ulcer just distal to the pylorus and two dogs developed duodenal erosions with marked antral gastritis. The other dog showed a mild antral gastritis.

It would appear that these latter 2 procedures offer relatively satisfactory protection against the development of ulcer but not as complete as the extensive gastric resection.

Conclusions. The extensive gastric resection (operations III and IVA) offer the best protection against the development of jejunal ulcer. No jejunal ulcer developed and all dogs survived the injection period.

Gastrojejunostomy, the small gastric resection and the Schmilinsky operation offer no protection. All animals developed jejunal ulcer with employment of the histamine-beeswax technique.

The small gastric resection and the antral exclusion operations would suggest that the Edkins' "gastrin" hypothesis is invalid.

14667

Morphogenetic Effects of Ultraviolet Radiation.

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Holtfreter has recently described[1] the physical properties of the superficial membrane (the "surface coat") of the early Amphibian embryo, and has indicated[2] ways in which it may play an important role in morphogenesis. Ultraviolet radiation would seem an excellent means for further study of the surface coat, since this type of energy penetrates poorly, and methods have been worked out which permit its localization in any given region of the embryo.[3]

In surveying the literature on the effects of ultraviolet radiation on embryonic cells, a number of discrepant observations become apparent. On the whole, ultraviolet exerts an inhibitory effect, but various stimulatory effects have been described, for which it is difficult to account. Thus Hinrichs[4] found various degrees of inhibition in *Fundulus* embryos, but a few were stimulated, presumably because they were affected by only a small dose of energy. In a general discussion Hinrichs[5] later asserts that although vertebrate embryos may be stimulated with ultraviolet, this is to be done only with difficulty. Higgins and Sheard[6] obtained a transitory speeding up of gastrulation in *Rana pipiens* eggs, but otherwise describe no stimulatory effects. Brandes[7] found that while most of his amphibian embryos showed inhibitory effects on the radiated side, others were stimulated, showing more rapid gastrulation movements and increase in mass on the radiated side. As a preliminary to the further use of ultraviolet energy, the present study was carried out with the aim of ascertaining conditions under which consistent and reproducible effects may be obtained.

* The author is indebted to Mr. Alvin Sellers for aid in carrying out part of the work herein reported.

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6 Higgins, G. M., and Sheard, C., *J. Exp. Zool.*, 1926, **46**, 333.

7 Brandes, J., *Bull. Acad. Roy. Belg. (Bull. de la Classe des Sciences)*, 1938, **24**, 92; *Ibid.*, Sceance du 5 Nov., 1938.

Over 600 eggs of the frog, *Hyla regilla*, were used. The egg-jelly absorbs ultraviolet energy very effectively therefore great care was taken to remove as much jelly as possible by rolling individual eggs in powdered carmine and then stripping off the carmine-jelly mixture by means of fine forceps.

Use of therapeutic cold quartz lamp. In a first group of experiments an instrument sold under the name of "Braun Fluoroscope" was used. A prospectus supplied with the instrument states that about 95% of its energy is emitted at λ 2537 Å. During radiation, the eggs were kept in a small quartz Petri-dish, 2.5 cm from the source of energy, and with just enough solution to cover them. Thermometers placed in the solution near the eggs showed no appreciable rise in temperature.

Late blastulæ and early and middle gastrulæ were irradiated. The blastulæ were first stained on one side of the presumptive epidermal region with nile blue sulfate and then the opposite (unstained) side was irradiated. Gastrula were treated on the right or left side without use of vital stain. Exposure times, in minutes, were: ¼, ½, 1, 1¼, 1½, 1¾, 2, 2¼, 2½, 2¾, 3, 4, 5, 7, and 10. After treatment the eggs were removed from the quartz chamber to stender dishes containing 0.30% Ringer solution and observed at various intervals during a period of 8-10 days. As criteria for stimulation or inhibition the following aspects of development were compared on the radiated and non-radiated sides of the embryo: (1) The progress of gastrulation, (2) formation and closure of the neural plate, (3) development of the gill filaments and adhesive glands, (4) curvature of the body, (5) length of body and tail.

Results. No effects on development could be observed in eggs exposed for less than 2 minutes. This is doubtless to be attributed to absorption of the ultraviolet by the thin layer of egg-jelly and by the chorionic membrane. On the exposed side of the egg the jelly is dissolved away completely and the chorion loses its toughness and elasticity. Near the end of gastrulation the embryo must be removed from the membrane in order to permit the elongation of the embryo, which becomes pronounced in the tail-bud stage. Embryos which are allowed to remain within the irradiated membrane, curl upon themselves as a result of the diminished elasticity of the membrane, and thus the effects produced by irradiation may be complicated or obscured by secondary effects due to cramped quarters.

Eggs exposed for 2 minutes or longer showed a distinct difference between the radiated and non-radiated sides. In every instance the developmental processes were inhibited on the radiated side. The gastrulation movements were retarded so that a persistent yolk-plug was a common feature. The appearance of the neural fold was retarded and, in many cases, absent on the radiated side. The gill filaments and adhesive glands developed more slowly on the radiated side. The total body length was decreased but this was largely due to stunting of the tail rather than of the body proper. The unusual sensitivity of the tail to ultraviolet radiation is evident in many specimens and is of especial interest in view of the outstanding sensitivity of the tail to centrifugal force.[8]

In every case examined, in a total of 119 early gastrulæ which showed a distinct curvature of the body, the concave side corresponded to the side previously radiated.

Use of ultraviolet monochromator.† In this apparatus a high pressure quartz mercury arc is the source of energy and monochromatic ultraviolet light is obtained by use of fused quartz prisms and lenses. The lines of light were isolated by means of a slit in a sheet of metal and were thrown upon the right or left side of the early gastrula, which was placed close to the wall of a quartz chamber. The following lines were used, in Angstrom units: 3660, 3130, 3020, 2804, 2654, 2537, 2350. Preliminary experiments showed that no discernible effect could be produced by exposures shorter than 2½ minutes. The exposure time of 2½ minutes is evidently very close to the

[8] Schechtman, A. M., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 430.

† We are indebted to Dr. Phillip A. Blacet of the Department of Chemistry for the use of this apparatus. For description of the monochromator, see Blacet, P. A., and Blacet, F. E., *J. Am. Chem. Soc.*, 1932, **54**, 3165.

minimal effective exposure since about 27% of radiated eggs were unaffected. Eggs radiated for 3 or 4 minutes showed very clear inhibitory effects. Of the various wave lengths listed above, all except 3660 showed some inhibitory effect. Somewhat more pronounced abnormalities of development occurred in the group of eggs exposed to the line at 2537, but most of the eggs showed no greater degrees of inhibition than can be found in eggs exposed to the other effective wave lengths. There is so much variation in any one group that conclusions as to relative effectiveness of various wave lengths under the present experimental conditions seem impossible. Variations in the amount of jelly which remains attached to the chorion and, possibly, variations in the degree of pigmentation of various eggs may account for the differences observed.

In this group of eggs, as in those treated with the therapeutic lamp, we have consistently observed inhibitory effects only.

Explanted material. Two types of explants (presumptive ectoderm and dorsal plastoporal lip) were removed from early gastrulæ, irradiated on both sides, and then cultured in Holtfreter solution. Non-radiated presumptive ectodermal explants normally round up, form a closed vesicle, and move over the substrate by ciliary action. Non-radiated dorsal lip explants normally elongate, forming curved finger-like structures. After radiation the explants round up more slowly than controls, or they may not do so at all. They have a strong tendency to stick to the surface of the quartz chamber, and cast off disintegrated material after they have been returned to culture dishes. As a consequence, the radiated specimens are frequently smaller than the controls. The radiated ectodermal explants move about the culture dish more slowly than controls or may be altogether quiescent. Many of them form small compact balls with little or no central cavity.

The dorsal lip explants are especially worthy of notice since their degree of elongation permits easy comparison between radiated and non-radiated specimens. After exposure for ¼ and ½ minutes only a slight or doubtful shortening is observed in radiated specimens. Those treated for ¾, 1, 1½, 2, and 3 minutes are consistently shorter than controls, while those treated 4, 6, and 10 minutes do not elongate at all, or form only a short nipple-like protuberance. Disintegrated fragments are commonly present in the culture dishes of irradiated specimens.

Effect of radiation upon inductive capacity. Four groups of dorsal lip explants (10 explants per group) were irradiated on both sides, their disintegrated fragments removed by passing through 3 dishes of Holtfreter solution, and then implanted into the blastocoeles of early gastrulæ. The 4 groups were exposed to the therapeutic lamp for 2, 4, 6, and 10 minutes respectively. (Total exposure was double the values given since the explants were treated on both sides). Arranging the specimens in the order of increasing exposure to radiation, neural plates were induced in 9 of 10, 8 of 10, 9 of 10, and 6 of 10 specimens in the 4 groups. In the last group (irradiated 10 minutes), 4 of the implants were expelled from the host embryo, so that actually there was 100% neural plate induction in the successful specimens. The neural plates induced by these irradiated explants were, taken as a whole, smaller than those induced by non-irradiated implants. This is especially clear in the case of explants radiated for 6 to 10 minutes. These results are contrary to the conclusions reached by Durken,[9] but quite in line with the results obtained by Reith.[10]

Summary. Entire eggs and explants from the portions of the egg of a frog (*Hyla regilla*) were irradiated for various periods of time using a (1) therapeutic cold quartz lamp and (2) an ultraviolet monochromator. Only inhibitory effects were exerted upon the several morphogenetic processes under observation.

[9] Durken, R., *Z. Wiss. Zool.*, 1936, **147**, 295.

[10] Reith, F., *Z. Wiss. Zool.*, 1937, **150**, 179.

14668

Prolongation of Hyperglycemic Effect of Epinephrine in Rabbits by Addition of Zinc Chloride.

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In the course of studies related to the prolongation of the action of subcutaneously injected medicines in man (Foldes[1]), it became desirable to investigate certain factors which influence the prolonging effect of the admixture of $ZnCl_2$ to these substances. The hyperglycemic response of rabbits to subcutaneous epinephrine seemed suitable for such investigation. That the addition of metal salts prolong the hyperglycemic activity of epinephrine has already been shown by Schwab[2] whose experiments served as a basis for the following observations.

Methods and material. Twelve rabbits, both male and female, were used for the experiments. Since the hyperglycemic response varied greatly, not only in different animals, but also in the same animal on different occasions, at least 4 animals were used for any single observation and each experiment was repeated on the same animal. An interval of at least one week was allowed to elapse between 2 epinephrine administrations on the same animal. The animals were kept in separate cages on uniform diet (oats, fresh green vegetables). Food and water were withheld for 18 hours before the experimental period.

Epinephrine hydrochloride was freshly prepared from epinephrine base on each occasion and ZnCl2 was added from a concentrated solution just before injection. The pH of the solution injected was around 5.5, its epinephrine content 0.1% and its Zn^{++} concentration varied from 0.0% to 2.0%. The dose of epinephrine was 0.03 mg per kg of body weight. Larger doses were used only in a few instances, since they were apt to produce blood sugar values above 400 mg %. The determination of blood sugar values in this range, using the Hagedorn-Jensen method[3] employed in these experiments has been found difficult. The injections were made subcutaneously either in the nuchal or in the gluteal region. Care was taken to avoid intracutaneous injection of the material. For sugar determinations blood was taken in duplicate from the ear veins before and ½, 1, 2, 3, and 4 hours after the injections.

Results. In preliminary experiments it was found that the prolonging effect depends on the Zn^{++} concentration of the injected solution and on the site of the injections. In rabbits Zn^{++} concentrations below 0.4% produce no noticeable effect. As the concentration is increased the prolonging effect increases and reaches a maximum when the Zn^{++} concentration is 1.2%. On further increase the hyperglycemic effect decreases and finally it ceases altogether. All this is true only if the injections are made in a region with dense subcutaneous tissue, *e.g.*, in the gluteal region. If the injections are made into the loose subcutis of the nuchal region the results are different. Here a Zn^{++} concentration of 1.2% has practically no prolonging effect. This difference, depending on the site of the injection, can be seen from Fig. 1.

Studies with the solution containing Zn^{++} concentration of 1.2% were made on 12 rabbits. The results are presented in Fig. 2. It can be seen from the data presented that in the first half hour following the injection of epinephrine alone, or epinephrine plus zinc, the hyperglycemic effect is practically the same. From there on, however, there is a marked difference in the behavior of the two curves. Following the injection of epinephrine plus zinc the maximum is reached

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1 Foldes, F. F., *J. Clin. Invest.*, 1943, **22**, 499.

2 Schwab, H., *C. R. Acad. d. sc.*, 1937, **205**, 628.

3 Hagedorn, H. C., and Jensen, B. N., *Bioch. Z.*, 1923, **135**, 46.

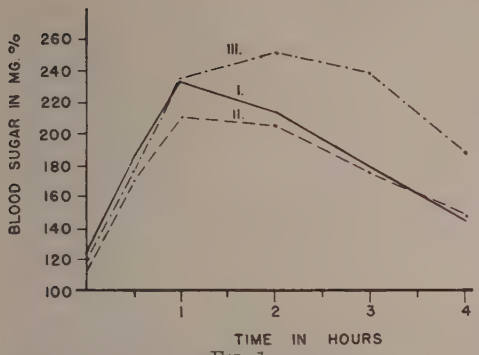

FIG. 1.

Combined blood sugar curves obtained on 4 rabbits: I. after the subcutaneous injection of 0.03 mg/kg of epinephrine alone in the nuchal region, II. after the subcutaneous injection of 0.03 mg/kg of epinephrine with 1.2% Zn^{++} in the nuchal region, and III. after the subcutaneous injection of 0.03 mg/kg of epinephrine with 1.2% Zn^{++} in the gluteal region.

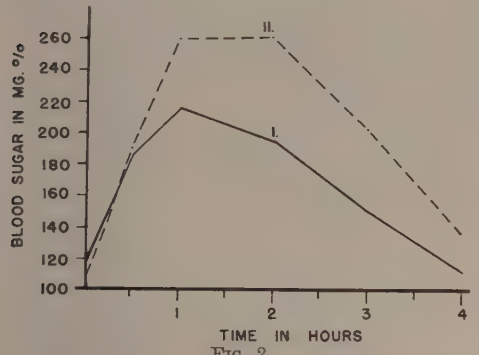

FIG. 2.

Combined blood sugar curves obtained on 12 rabbits: I. after subcutaneous injection of 0.03 mg/kg of epinephrine alone, and II. after subcutaneous injection of 0.03 mg/kg of epinephrine with 1.2% Zn^{++}.

later, the peak is higher, and the effect is more sustained, than after epinephrine alone. The absolute hyperglycemic effect, which can be measured by the surface area enclosed by the curve and 2 lines, one drawn through the starting point of the curve parallel with the X axis, and the other through the end point of the curve parallel to the Y axis, is 90% larger after the injection of epinephrine plus zinc, than after epinephrine alone. In a limited number of experiments similar results were obtained after the use of a 0.06 mg per kg dose. Not only was there a marked difference between the effect of this dose when administered alone or together with zinc, but the hyperglycemic effect of 0.06 mg per kg epinephrine plus zinc was superior to the hyperglycemic effect of 0.08 mg per kg epinephrine administered without zinc.

Comment. The experiments described corroborate the findings of Schwab in so far that a marked prolongation of the hyperglycemic effect of epinephrine has been observed after the admixture of Zn^{++}. Full comparison between Schwab's results and our findings cannot be made because of the lack of some data in his paper; namely, the number of rabbits used in his experiments is not stated; the dose of epinephrine and zinc injected is indicated but the concentration of the solution, which was found to be of great importance in the present work, is not given. The dose of epinephrine in his experiments was more than 8 times larger than our dose. The ratio between epinephrine and Zn^{++} was 25:15 in his experiments and 1:12 in the present study. Notwithstanding the much larger dose of epinephrine used by him the hyperglycemic response (as interpreted from the curves presented in his papers) obtained was only slightly different from those obtained by us with the much smaller dose. The main difference between his curves and ours is that the maximum of the hyperglycemic effect is reached one hour later, both after the injection of epinephrine alone and epinephrine with Zn^{++}.

That higher blood sugar levels were attained after the injection of epinephrine plus zinc (the absorption of which is more delayed) than after the injection of epinephrine alone, on first sight seems paradoxical and therefore needs further explanation. It has been known that epinephrine is rapidly destroyed in the organism by the action of "amine oxidase" (Blaschko, *et al.*[4]) If the absorption of epinephrine is not delayed, then it enters the circulation more quickly and consequently is destroyed before it has time to mobilize liver glycogen to the fullest extent. For the development of the hyperglycemic effect, the presence of relatively small amounts of epinephrine over a longer period of time is of more importance than the pres-

[4] Blaschko, H., Richter, D., and Schlossmann, H., *J. Physiol.*, 1937, **90**, 1.

ence of larger amounts over a shorter period of time. Thus, it was found (Cori[5] *et al.*) that the continuous intravenous infusion of 0.00025 mm of epinephrine per minute produced maximal effect and that doubling or quadrupling of this amount produced no increased effect.

It is also well known (Cori[6]) that the intravenous injection of relatively large amounts of epinephrine is followed by hyperglycemia of short duration. By prolonging the absorption of epinephrine through the addition of zinc, epinephrine enters the circulation at a rate which is better for its economical utilization. This explains that epinephrine plus zinc not only has a longer lasting effect but that the maximum of this effect is also higher than when epinephrine is injected alone.

Summary. 1. The hyperglycemic effect of epinephrine in rabbits can be prolonged and increased by the admixture of Zn^{++} (in the form of $ZnCl_2$). 2. The prolonging effect of Zn^{++} depends on the concentration of the injected solution and the site of the injection. 3. Optimal results were obtained when the Zn^{++} concentration of the injected solution was 1.2% and the injections were made in the subcutis of the gluteal region.

5 Cori, C. F., Cori, G. T., and Buchwald, K. N., *Am. J. Physiol.*, 1930, **93**, 273.

6 Cori, C. F., *Physiol. Rev.*, 1931, **11**, 143.

14669

Relative Sensitivity of Different Phases of Growth Curve of *Bacterium salmonicida* to Alkaline Acriflavine.

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The action of acriflavine solutions on *Bacterium salmonicida* has been the object of several studies[1,2,3] because of its efficacy in the prophylactic disinfection of the exterior of trout eggs to prevent the spread of *Bacterium salmonicida* infection (trout furunculosis).

None of these reports has recorded the action of organisms tested in the various phases of the growth curve. Furthermore, a search of the literature has revealed no data on the relative sensitivity to lethal agents of the growth phases of an organism grown near its optimum temperature as compared to organisms grown near the minimum.

Using a modification[3] of the method of Ruehle and Brewer[4] for testing disinfectants, *Bact. salmonicida* cultures incubated for various periods of time and at 2 different temperatures (15°C and 10°) were exposed to the action of 500 parts per million (p.p.m.) of alkaline acriflavine.* The tests of the 15° cultures were run at pH 7.7, those grown at 10° at pH 8.0. The disinfection studies were run at 10°.

Table I summarizes the tests and Fig. 1 presents the death curve of the cultures.

The cultures grown at 15° showed little variation. There was a slight but definite increase in resistance to the dye as the culture aged. A peculiarity (autoagglutination) of the test organism prevented the procurement

1 Blake, Isobel, *Fisheries, Scotland, Salmon Fish*, 1930, No. 2, His Majesty's Stationery Office, Edinburgh, 1933.

2 Gee, Lynn L., and Sarles, W. B., *J. Bact.*, 1942, **44**, 111.

3 Smith, Winslow Whitney, PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 324.

4 Ruehle, G. L. A., and Brewer, C. M., U. S. Dept. Agri., Circ. 198, 20 pp., Washington, D.C., 1931.

* Abbott Laboratories Acriflavine lot No. 539126 used in all tests.

TABLE I.
Influence of Phase of Growth Curve on Sensitivity of *Bacterium salmonicida* to Acriflavine.

Days since subculture	1/24	1	2	3	4	5	6	7	8	9	10	12	14	16	18	21	24	27
Minutes to kill culture grown at 10°C	10	9.3	7.9	8	6.2	10	7.5	8.7	8.3	8.3	10	10	10	18	15	15	5	5
Minutes to kill culture grown at 15°C	5	7.5	5	5	7	5	5	8.2	5	8.2	6	7	7	8	7.5			

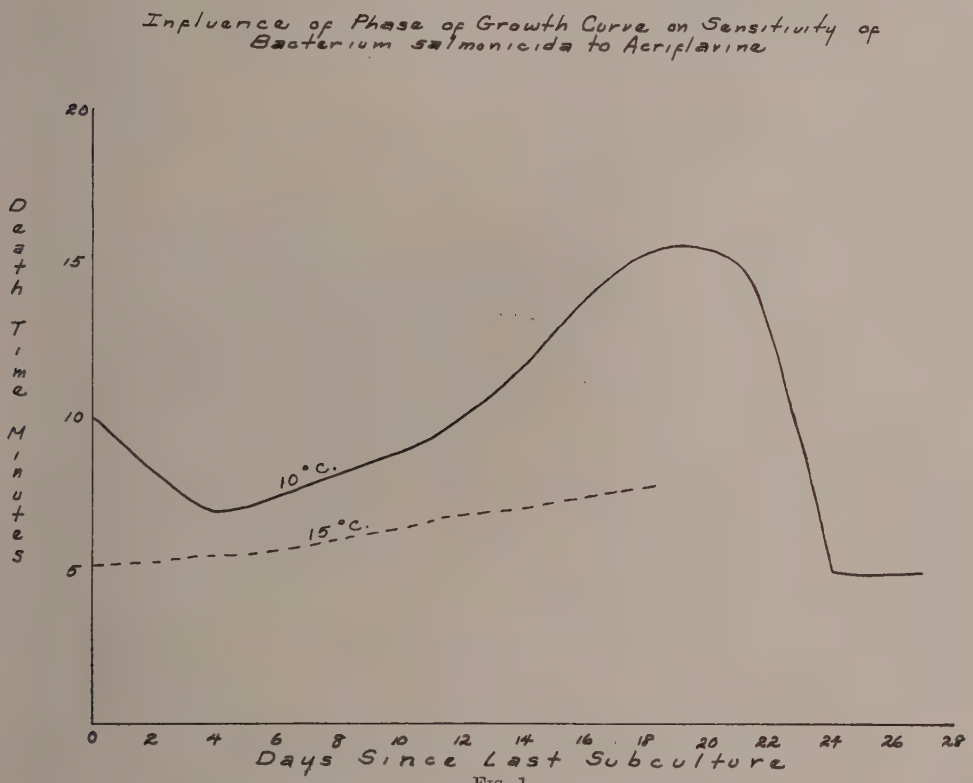

FIG. 1.

of bacterial counts. Therefore, it was not possible to compensate for the variations in load of organisms at each time interval. Had this been possible the death time of the youngest and oldest cultures would probably have been prolonged. The curve of death would have been U-shaped.

The 10° culture showed greater variation with age than the 15° culture. In what is assumed† to be the phase of exponential growth the organism was killed more rapidly than in earlier or later phases except that after 24 days all cultures were killed in 5 minutes. It is believed that had it been possible to correct for variations in load this curve would have shown a much deeper U

† The various phases of the growth curve cannot be positively identified. Thirty-eight different attempts to count the organisms in the various phases of the growth curve both by plate and direct count failed to yield conclusive data. There is, however, some indication that at 10° the lag phase ends in 5 hours, and the logarithmic growth phase in 48 hours. In 12 days the organisms were definitely in the phase of "logarithmic" death. The growth at 15° was faster.

shape than it does. Consequently, the 10° curve would have shown even greater relative variation when compared to the 15° culture. It is possible that the short death time of the 24- and 27-day cultures can be explained on the basis of reduced load of organisms tested.

It must be pointed out that since the 10° culture was studied at pH 8 and the 15° at pH 7.7 these 2 curves can be compared only as to shape, not as to magnitude.

The 2 incubation temperatures are believed to represent the optimum and the minimum because, of cultures grown at 5°, 7½°, 10°, 12½°, 15°, 17½°, and 20°, the 15° tubes clouded most rapidly. The 7½° showed no clouding.

These data indicate that for *Bact. salmonicida*—a Gram-negative psychrophile—organisms in the lag phase and the "logarithmic" death phase are more resistant to the action of 500 p.p.m. alkaline acriflavine solution at 10° than are bacteria in the phase of logarithmic growth. Organisms grown below the optimum temperature show a greater variation than those grown at the optimum.

14670

Production of Unidentified Vitamins by a Strain of *Mycobacterium tuberculosis* Grown on Synthetic Medium with p-Aminobenzoic Acid.*

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Mayer[1] reported the formation of a yellow pigment in cultures of a certain strain of Mycobacterium tuberculosis when grown in the presence of high concentrations of p-aminobenzoic acid (PABA). He suggested that the pigment might possibly be related to factors of the vitamin B complex. Since the solubility properties of the pigment were similar to those of vitamins B_{10} and B_{11}[2] which promote feathering and growth in chicks, and since PABA can partially replace these vitamins in chick diets,[3] it was decided to feed a culture filtrate of the organism grown in the presence of PABA to chicks on a diet deficient in the above vitamins.

The chick ration used (493K) contains, per 100 g: dextrin 61 g, alcohol extracted casein 18 g, gelatin 10 g, salts V[2] 6 g, soybean oil 5 g, l-cystine 0.3 g, choline 0.15 g, inositol 0.1 g, biotin 20γ, thiamin 0.3 mg, pyridoxine 0.4 mg, riboflavin 0.6 mg, pantothenic acid 2 mg, nicotinic acid 5 mg, 2-methyl-1, 4-naphthoquinone 50γ, and alpha-tocopherol 0.3 mg. Vitamins A (1200 I. U.) and D (120 I. U.) were fed by dropper weekly. Water and the experimental ration were given *ad libitum*. On this ration chicks grew slowly and feathered poorly, as shown in Table I. The addition of 2% liver fraction L (solubilized liver) or the Super filtrol eluate of liver fraction L[4] provided for rapid growth and good feathering.

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

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We wish to thank Miss Janet R. McCarter, Department of Agricultural Bacteriology, University of Wisconsin, for the culture used; Merck and Company, Rahway, N.J., for the crystalline vitamins; Wilson Laboratories, Chicago, Ill., for the liver fraction L; and Wilson and Co., Inc., Chicago, Ill., for the gelatin.

1 Mayer, R. L., *Science*, 1943, **98**, 202.

2 Briggs, G. M., Jr., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1943, **148**, 163.

3 Briggs, G. M. Jr., Luckey, T. D., Mills, R. C., Elvehjem, C. A., and Hart, E. B., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 7.

4 Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, 1941, **141**, 521.

TABLE I.
Growth Results with Chicks.

Supplement to Ration 493K	No. of chicks	Avg wt at 4 weeks g	Feathers 0 = very poor 100 = very good	"Folic acid"* supplied/100 g ration, γ
None	11	145	33	0
Super Filtrol eluate of liver fraction L ≅ 5%	12	260	98	50
Norite eluate of culture filtrate ≅ 55 cc/100 g	6	230	85	16
" " " " " (no PABA) ≅ 80 cc/100 g	6	196	45	4
Band No. 1 of norite eluate ≅ 80 cc/100 g	6	130	30	0.4
" " 2 " " " ≅ 80 cc/100 g	6	184	50	7
" " 3 " " " ≅ 80 cc/100 g	6	245	85	5
Dialysis filtrate of norite eluate ≅ 80 cc/100 g	5	234	80	12
" residue " " " ≅ 80 cc/100 g	6	188	40	2.5

* "Folic acid" is measured with *Streptococcus lactis*.[6]
Figures are based on a standard having a potency of 40,000.[7]

In the bacterial work we employed the same organism that Mayer used, Strain No. 607 of the American Type Culture Collection. It was grown on Long's medium[5] plus 1 g PABA per liter. Long's medium contains per liter: asparagine 5 g, ammonium citrate 5 g, K_2HPO_4 3 g, Na_2CO_3 3 g, NaCl 2 g, $MgSO_4$ 1 g, ferric ammonium citrate 0.05 g, and glycerol 50 g. It is made up to volume with water and adjusted to pH 7.

The procedure for preparing the norite eluate of the culture filtrate was as follows: 4 liters of the medium were divided among 12 one liter Erlenmeyer flasks, which were then plugged, sterilized, inoculated, and incubated at 37° C for 14 days. After a few days growth the medium began to turn yellow, and later became brown. The flasks were autoclaved and the contents filtered through a Super Cel pad on a Buchner funnel. The filtrate (3300 cc) was adjusted to pH 3 with H_2SO_4 and the precipitate filtered off and discarded. Twenty-five g of Norite A were then added, the mixture stirred for 75 minutes and the norite filtered off. The filtrate was discarded. The norite was treated with 200 cc of elution mixture (50 cc ethyl alcohol, 10 cc NH_4OH, and water to 100 cc) for 45 minutes, filtered, and the elution repeated once. The combined eluates were concentrated *in vacuo* to 100 cc and made to pH 4. They were then extracted 20 times in a separatory funnel with 50 cc portions of ethyl ether, to remove the remaining PABA, and the residue was made alkaline to litmus with NH_4OH.

When the norite eluate, prepared as described above, was fed to chicks at a level equivalent to 55 cc of the original filtrate per 100 g of ration, good growth and feathering resulted, approaching that obtained with the Super Filtrol eluate of liver.

In an attempt to further purify the active material, a portion of the norite eluate was made to pH 5 and passed through an adsorption column of Super Filtrol and Super Cel (2:5), 2.5 x 50 cm. After developing with distilled water, 3 distinct yellow-brown bands were obtained, which were separated, eluted with the elution mixture, concentrated, and fed at a level equivalent to 80 cc of original filtrate per 100 g of ration. The results in Table I show that most of the activity was in band No. 3, the lower and least strongly adsorbed band. This band supplied 50 mg of dry matter per 100 g of ration, as compared with 100 mg supplied by the Super Filtrol eluate.

Another portion of the norite eluate was dialyzed through a Cellophane tube (Visking No. 232), and it was found that most of the activity passed through the tubing, as has been found to be true with vitamins B_{10}, B_{11} and "folic acid" with this tubing.

[5] Wells, H. G., and Long, E. R., *The Chemistry of Tuberculosis*, 2nd Ed., 1932.

[6] Luckey, T. D., Briggs, G. M., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **152**, 157.

[7] Mitchell, H. K., and Snell, E. E., *Univ. Texas Pub.*, 1941, **4137**, 36.

As a control, a culture was grown in Long's medium without added PABA, and the norite eluate was prepared as described above. When it was fed at a level equivalent to 80 cc of the original filtrate per 100 g of ration, the growth and feathering obtained were somewhat better than on the basal ration, but did not approach the optimum.

The amount of "folic acid" supplied by the various preparations per 100 g of ration is also shown in Table I. Although in most cases there was fair correlation between the "folic acid" content of the ration and growth and feathering, band No. 3, which gave good results, supplied only 5γ of "folic acid" per 100 g of ration, less than that supplied by band No. 2. This indicated that the response of the culture filtrates could not be attributed to their "folic acid" content.

These results show that compounds active for the chick are synthesized by a strain of *Mycobacterium tuberculosis* on a completely synthetic medium, and that the production of these vitamins is definitely stimulated by high concentrations of PABA in the medium. This observation is interesting in light of our earlier suggestion[3] that feeding PABA to chicks on the basal ration causes increased growth by stimulating the production of the unknown vitamins by the intestinal bacteria. This increased production of these vitamins may be a result of actual incorporation of the PABA into the vitamin molecules, or of stimulation of the bacterial metabolism in some manner to produce compounds distinctly different from the PABA.

Summary. Culture filtrates of a certain strain of *Mycobacterium tuberculosis* grown on a synthetic medium containing high concentrations of *p*-aminobenzoic acid, contain considerable amounts of the unidentified vitamins (B_{10} and B_{11}) which promote growth and feathering in chicks. The synthesis of these vitamins by the microorganism is definitely stimulated by the presence of high concentrations of *p*-aminobenzoic acid in the medium.

14671 P

Effects of Desoxycorticosterone Acetate on Water and Electrolyte Content of Brain and Other Tissues.

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In a previous study on the antagonistic action between pitressin and desoxycorticosterone acetate in epileptic subjects, the authors[1] found the latter substance to have a striking anti-convulsive effect for both spontaneously occurring and pitressin-induced seizures. The principal experimental subject involved in that investigation, a young man with extremely severe epilepsy, has remained essentially free from convulsive attacks during the intervening 3 years while continuing to receive this synthetic hormone sublingually or by subcutaneous pellet implantation. The present experimental study was undertaken with the hope of obtaining some information regarding the physiological or pharmacological mechanism responsible for this effect.

Our immediate objective was to determine the effects of the hormone on the water and electrolyte content of the brain tissue of normal animals. It has been abundantly demonstrated by Darrow and Miller[2,3] and by Ferrebee and co-workers[4] that the potas-

[1] McQuarrie, I., Anderson, J. A., and Ziegler, M. R., *J. Clin. Endocrinol.*, 1942, **2**, 406.

[2] Miller, H. C., and Darrow, D. C., *Am. J. Physiol.*, 1941, **132**, 801.

[3] Darrow, D. C., and Miller, H. C., *J. Clin. Invest.*, 1942, **21**, 601.

[4] Ferrebee, J. W., Parker, D., Carnes, W. H., Gerity, M. K., Atchley, D. W., and Loeb, R. F., *Am. J. Physiol.*, 1941, **135**, 230.

TABLE I.
Effects of Desoxycorticosterone on Water and Electrolytes of Brain and Muscle Tissue.

Tissue	H_2O g	Cl m. eq.	K m. eq.	Na m. eq.
	Per kilo blood-free wet tissue			
Brain				
Controls	776	33.72	77.30	49.73
"Doca"	776	33.24	60.58	52.45
Muscle				
Controls	750	11.84	75.43	31.98
"Doca"	751	12.90	60.67	33.65
	Per 100 g fat-free solids			
Brain				
Controls	605	26.28	60.25	38.75
"Doca"	602	25.77	46.96	40.66
Muscle				
Controls	341	5.38	34.28	14.53
"Doca"	335	5.75	27.08	15.02

sium content of skeletal muscle, as well as that of blood plasma, is greatly reduced and the sodium content is somewhat increased by daily injections of comparatively large doses of desoxycorticosterone acetate. Heart muscle and liver showed much less, or (in some animals) no alteration.[3] Data regarding the brain were not reported by these authors. In the present study, changes in skeletal muscle, liver and heart muscle were determined for the purpose of comparison.

Eighty young hooded rats (initial weights, 150 to 200 g each) maintained continuously from the time of weaning and throughout the experiments on a standard rat diet containing 0.605 g of K, 0.565 g of Na, and 1.155 g of Cl per 100 g, were divided into two equal groups. The 40 experimental animals were each given daily doses of 1 mg of desoxycorticosterone acetate, one sub-group receiving it for a period of 7 days only, others for 23, 28, 35, and 41 days respectively. This was administered subcutaneously in sesame oil. The 40 control animals were subdivided into similar groups and were treated identically except for the omission of the steroid from the oil injected.

At the expiration of the various periods, the rats were decapitated and thoroughly bled without anesthesia. Fresh samples of the different tissues were then dissected out and immediately placed in stoppered weighing bottles. Aliquot portions of the minced tissues were analyzed for the following constituents: *water*, by drying in oven at 105°C; *total lipids* by first extracting with alcohol and ether and then with petroleum ether; *hemoglobin* (method of Flink and Watson[5])—for use in calculations of extracellular constituents; *potassium* (Shohl and Bennett[6]); *sodium* (Butler and Tuthill[7]), and *chloride* (Wilson and Ball modification of Van Slyke method.[8]). The relative masses of extra- and intra-cellular phases of the tissues were calculated according to the method of Manery and Hastings.[9]

The results are summarized in Table I. Since variations in the effects of desoxycorticosterone acetate due to differences in length of periods of administration were insignificant, the values given represent the averages for all of the experimental animals on the one hand and for all of the controls on the other.

It is apparent from the data presented that desoxycorticosterone acetate caused a reduction of approximately 20% in the K content of the brain tissue, which was almost identical with the reduction found by previous workers and by ourselves in the case of skeletal muscle. The sodium was but slightly increased in both tissues. While heart muscle showed no decrease in K content, liver was found to

[5] Flink, E. B., and Watson, C. J., *J. Biol. Chem.*, 1942, **146**, 171.

[6] Shohl, A. T., and Bennett, H. B., *J. Biol. Chem.*, 1928, **78**, 643.

[7] Butler, A. M., and Tuthill, E., *J. Biol. Chem.*, 1931, **93**, 171.

[8] Wilson, D. W., and Ball, E. G., *J. Biol. Chem.*, 1928, **79**, 221.

[9] Manery, J. F., and Hastings, A. B., *J. Biol. Chem.*, 1939, **127**, 657.

suffer a 30% loss in this element as a result of the steroid injections. There was no significant alteration in the water content of the brain or other tissues examined. Whether or not the anti-epileptic effect of desoxycorticosterone acetate previously referred to is in any way related to the observed decrease in brain potassium cannot be determined on the basis of our present knowledge.

14672

Effect of Low Fat Diet on Lipids of Erythrocytes, Plasma, Serum, and Whole Blood of Dogs.*

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Although many reports have been made concerning the relative amounts of lipid in plasma and red blood corpuscles in both human beings and lower animals,[1-8] relatively little information is available concerning the qualitative characteristics of the fatty acids involved, especially as these may be affected by dietary conditions. Hansen and Wiese[9, 10] have reported that the fatty acids in the various lipid fractions of the serum were definitely less unsaturated in dogs maintained on diets extremely low in fat as compared with those found in control animals receiving lard as the source of dietary fat. The study here reported concerns the character of the fatty acids in the red blood cells in dogs and the effect of dietary fat on these fatty acids, as well as on those in the plasma, serum and whole blood.

Material and Methods. Six dogs, 3 on a low fat diet and 3 on the same diet except for the isocaloric substitution of 28% of lard for sucrose, were maintained under conditions described by Hansen and Wiese.[9] Blood was drawn from the jugular vein after a fast of 16-20 hours. The anticoagulant used was a saturated solution of ammonium oxalate, 0.024 cc per cc of blood. In the determination of the lipids of the erythrocytes, about 20 volumes of the usual 3:1 alcohol-ether mixture of Bloor were added rapidly to the red blood cells which had been laked with an equal volume of distilled water. The mixture was agitated, then heated in a boiling water bath. At times a solid gummy mass formed. This was ground with sea sand and returned to the extraction mixture. Simultaneous determinations indicated that the results were the same whether or not this mass formed. The extract was filtered through lipid-free filter paper into a volumetric flask, numerous additional extractions were made, the solution was cooled to room temperature and brought to volume. For the extraction of the lipids of whole blood, 3 cc of the oxalated blood were added slowly to the hot alcohol-

* Aided by a grant from the National Live Stock and Meat Board through the National Research Council.

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4 Iwatsura, R., *Arch. f. d. ges. Physiol.* (Pflüger's), 1924, **202**, 194.

5 Boyd, Eldon M., *Am. J. Dis. Child.*, 1936, **52**, 1319.

6 Bloor, W. R., *Biochemistry of the Fatty Acids and Their Compounds, the Lipids*, New York, 1943, Reinhold Publishing Corporation.

7 Erickson, B. N., Williams, H. H., Hummel, F. C., and Macy, I. G., *J. Biol. Chem.*, 1937, **118**, 15.

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9 Hansen, Arild E., and Wiese, H. F., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 205.

10 Wiese, Hilda F., and Hansen, Arild E., *Fed. Proc.*, 1944, **3**, 97.

TABLE I.

Comparative Values for Total Fatty Acids Including Amount, Iodine Number and Average Molecular Weight Found in Erythrocytes, Plasma, Serum, and Whole Blood in 3 Dogs Reared on a Low Fat Diet and 3 Dogs on Control Diet with Lard as the Source of Fat.

Animal No.	Date	Hemato-crit	Erythrocytes			Plasma			Serum			Whole Blood		
			mg%	I.N.	M.W.	mg%	I.N.	M.W.	mg%	I.N.	M.W.	mg%	I.N.	M.W.
Animals on Low Fat Diet.														
19	10-13-42	51.6	235	100.1	—	490	95.4	—	440	94.4	—	334	99.2	—
20	10-20	47.0	295	105.8	285	447	96.5	281	402	96.3	290	324	100.0	284
19	11- 5	52.5	239	106.0	293	473	92.6	286	424	92.2	284	344	102.1	286
20	11-17	51.2	226	105.5	283	494	95.6	291	454	94.3	290	330	102.9	288
20	4- 6-43	52.3	290	98.6	297	*470	89.0	290	—	—	—	—	—	—
26	4-27	45.7	281	101.9	298	*416	91.3	291	—	—	—	—	—	—
Avg			261	103.0	291	465	93.4	288	430	94.3	288	333	101.1	286
Control Animals Receiving Lard in the Diet.														
17	10-13-42	53.8	219	117.5	—	428	110.6	—	385	110.9	—	312	113.3	—
15	10-20	52.5	242	109.8	284	505	115.4	285	461	115.3	284	358	114.9	286
17	10-29	55.7	235	111.6	286	582	106.9	286	514	107.1	287	364	112.0	279
15	11-10	57.2	247	114.4	—	640	116.9	287	559	117.0	—	390	116.1	281
17	4- 6-43	60.1	248	110.0	284	*414	105.7	285	—	—	—	—	—	—
24	4-27	47.1	213	120.5	288	*480	114.5	—	—	—	—	—	—	—
Avg			234	114.0	286	508	111.7	286	480	112.6	286	356	114.1	282

* Plasma removed after 10 minutes' centrifugation.

ether mixture. Plasma and serum extracts were prepared in the usual manner. Methods of analysis were those used by Hansen and Wiese,[9] except that alpha-naphtholphthalein was used in place of phenolphthalein in the molecular weight determinations. The results of the analyses are given in Table I.

Discussion. The majority of the authors reporting studies on lipid values for blood plasma and red blood cells[1-8] have found considerable variation in the quantity of the former, but a relative constancy in that of the latter. In our studies, the level of the blood fat in neither the erythrocytes, plasma, serum nor whole blood appeared to be greatly influenced by the amount of fat in the diet (Table I). As a matter of fact, there was more variation in this respect within each of the animal groups and in individual animals at different times than there was between the two groups. The quantity of total fatty acids per unit of erythrocytes was definitely lower than that per unit of plasma or serum. The unexpected and consistent variation between the plasma and serum fat levels was shown to be due to evaporation of water from the plasma during the relatively long process of centrifugation. The average molecular weight of the fatty acids of the cells was essentially the same as that of the other portions, indicating no difference in the length of their carbon chains.

As regards the effect of the diet on the qualitative characteristics of these fatty acids, our studies revealed a higher degree of unsaturation in all 4 of the portions of the blood of the animals receiving fat than in those on the low fat diet, but the difference was not as marked in the erythrocytes as in the serum and plasma. It should be noted that in the determination of iodine numbers of blood fats, if whole blood or cells are used for the analysis, differences in iodine numbers of the fatty acids may not appear significant. Epstein and Glick[11] were unable to note any difference in iodine numbers of blood lipids whether they used whole blood or serum, but our findings indicate that this is not true when abnormal iodine values are present. The finding that the characteristics of the fatty acids of the cells are not as greatly affected by the amount of fat in the diet as are those of the fatty acids of the serum and plasma is probably due in part to the fact that the cells contain relatively little of the cholesterol ester fatty acids, which in the serum have been shown to be the fraction most affected by

[11] Epstein, N. N., and Glick, D., *Arch. Dermat. and Syph.*, 1937, **35**, 427.

variation in the amount of fat in the diet.[9, 10] Even in the cells, however, this difference was notable, the iodine numbers in the low fat group ranging from 98-106 and those in the high fat group from 109.8-120.5, indicating that, although to not as great a degree as in the serum, the qualitative characteristics of the fatty acids of the erythrocytes can be affected by the diet.

In order to determine in which fraction of the lipids the greatest difference in the degree of unsaturation of the fatty acids existed, preliminary experiments were carried out with 4 animals (2 from each dietary group), wherein the fatty acids of the erythrocytes and plasma were separated into phospholipid, triglyceride and cholesterol ester fractions. Irrespective of the diet there was little variation noted in the degree of unsaturation of the fatty acids in the glyceride fraction of either the red cells or the plasma. On the other hand, the iodine values for the phospholipid and cholesterol ester fatty acids in both the red cells and plasma indicated that the character of these fatty acids was affected by the fat in the diet. The highest degree of unsaturation of the fatty acids in the erythrocytes was found to be in the phospholipid fraction, whereas in the plasma this was found in the cholesterol ester fraction.

Summary. From our studies of the fatty acids in the corpuscular and fluid portions of the blood, it appears that dogs on the low fat diet used in these experiments are able to synthesize sufficient fat to maintain a normal fat level in both the cells and the plasma but are unable to synthesize the highly unsaturated fatty acids necessary to maintain the degree of unsaturation normally found in both these portions. This confirms the previous conclusion of Hansen and Wiese[9, 10] from a study of the serum lipids.

SECRETARY'S REPORT

April 1, 1943-March 31, 1944

Change in Policy Concerning Length of Complete Manuscripts. The Board of Editors unanimously proposed a change in policy concerning length of complete manuscripts. The present length is limited to five printed pages. This space limitation has too frequently resulted in (1) dividing a research into a series of short manuscripts with obvious duplication; (2) submitting the by-product, the minor or "trivial" contribution; or (3) deterred authors from sending their primary contributions of interest to workers in borderline fields. The Editors recommended:

1. Continued policy of rigorous condensation.

2. Editors be permitted as in the past to determine space allotment.

3. *Complete manuscripts may be accepted up to ten printed pages in length, inclusive of tables and illustrations.*

4. In very exceptional instances only may Editors permit greater space.

The Council by almost unanimous vote approved this change in policy.

Editors. The chief burden of the Society falls on the overworked editors. They do a thankless job. Comments verbal or written often express the warm thanks of members for the meticulous, painstaking, and just comments of the editors. Rarely adverse or splenetic comments are made. May I, who am close to what is going on, pay my tribute to the editors for their splendid efforts, their fair appraisal, their devotion to the maintenance of reasonably high standards.

The Society is much indebted to Dr. Emil Baumann for his continued and excellent indices for each volume of the PROCEEDINGS.

Causes of delay in publication. The mails are now not as prompt as formerly. If authors would particularly note those items under "Instructions to Authors" which have an asterisk, they would expedite materially the publication of their manuscripts.

Surplus for the Year. In spite of increased costs of publication, postage, etc., the Society had a surplus of $4,774.58 (see Treasurer's report). This surplus was due primarily to the large number of subscriptions received from the United States Government for the army medical posts, to the sale of back numbers, and to the somewhat decreased number of manuscripts published.

Reduced Costs to Authors. In view of the present and past surpluses the Council approved the following reduced costs to authors:

1. Excess space to be reduced from 13 to 6½% of cost.

2. Illustrations to be reduced from 75 to 50% of cost. As before members are allowed 750 words a year without cost.

Treasurer's Accounts. The Council had approved the request that a certified public accountant be appointed to report on the accounts of the Treasurer. President Carlson appointed Doctors Detwiler, DuVigneaud, and Pappenheimer as auditors. These auditors appointed Mr. Alexander Dolowitz, C.P.A. His detailed report was submitted to the auditors and to our Finance Committee. His abbreviated report is given on page 250.

Finance Committee. During these difficult times, the investments of the Society were most carefully watched. Much thanks are due Doctors Lambert and Leake of the Finance Committee for their painstaking efforts. We are all very grateful to Mr. H. G. Friedman of the General Investors Company for continuing advice on investments, also to Mr. Edward Robinson of the Rockefeller Foundation, to Mr. Samuel Kramer for legal advice on matters pertaining to our guaranteed mortgages, and to Mr. William Girden for help on the non-legal phases of this difficult problem.

Texas Section. The Council approved a request for the formation of the Texas Section.

Members Emeritus. The following members, qualified for emeritus membership, were approved by the respective Sections and by the Council: E. L. Scott, C. A. Elsberg, L. Michaelis, W. E. Garrey, D. J. Davis, and F. A. McJunkin.

Resignations. Six resignations were approved by the Council with regret: H. Laurens, C. C. Lindegren, E. M. Greisheimer, H. S. Mitchell, J. Bonner, and W. W. Graves.

Members in Arrears. The Council is most reluctant to drop members for arrears. Those in the armed forces may retain their membership without payment of dues, upon due notification. Twenty-five members have availed themselves of this ruling.

Deaths of Members. The Council records with deep regret the death of the following members: Doctors H. G. Barbour, J. G. M. Bullowa, R. H. Chittenden, H. C. Coombs, B. Cunningham, J. Ewing, C. S. Gager, S. H. Geist, H. Koster, F. Ramaley, W. Salant, and L. T. Webster.

Gifts. The thanks of the Society are due the following members for the gift of back numbers of the PROCEEDINGS: Doctors A. P. Briggs, H. A. Charipper, M. B. Cohen, E. B. Cram, D. L. Fox, A. J. Geiger, S. C. Harvey, W. H. Howell, E. Ingersoll, J. A. Killian, M. O. Lipman, P. K. Olitsky, W. L. Palmer, I. H. Perry, E. D. Plass, W. C. Quinby, O. H. Robertson, E. A. Rovenstine, W. H. Seegers, E. L. Sevringhaus, R. R. Spencer, S. A. Thayer, S. L. Vaughan, E. D. Warner, Charles Weiss, H. S. Willis, E. Witebsky; and to the following libraries: Boston University, University of California, Medical College of South Carolina, University of Pennsylvania, Rockefeller Institute, University of Oregon, University of Texas, Yale University, Squibb Institute, Calco Chemical Company.

SECTIONAL MEETINGS AND MEMBERSHIP

Cleveland, Ohio

Chairman: N. L. Hoerr Secretary: R. W. Heinle Members: 45
Meetings: Western Reserve University, October 8, 1943
December 10, 1943
January 19, 1944
April 14, 1944

District of Columbia

Chairman: J. H. Roe Secretary: Henry Stevens Members: 77
Meetings: George Washington University, December 2, 1943
February 3, 1944
March 30, 1944
June 6, 1944

Illinois

Chairman: L. N. Katz Secretary: L. V. Domm Members: 149
Meetings: University of Chicago, November 9, 1943
March 14, 1944
May 23, 1944

Iowa

Chairman: W. M. Hale Secretary: E. D. Warner Members: 40
Meetings: State University of Iowa, November 18, 1943
February 10, 1944
May 11, 1944
May 25, 1944

Minnesota

Chairman: O. H. Wangensteen Secretary: W. D. Armstrong Members: 53
Meetings: University of Minnesota, January 19, 1944
March 15, 1944
May 17, 1944

Missouri

Chairman: A. S. Gilson, Jr. Secretary: L. R. Jones Members: 51
Meetings: St. Louis University, February 9, 1944

New York

Chairman: I. Greenwald Secretary: W. S. Root Members: 471
Meetings: New York Academy of Medicine, December 15, 1943
February 16, 1944
May 17, 1944

Pacific Coast

Chairman: F. W. Weymouth Secretary: M. Kleiber Members: 115
Meetings: University of California, July 31, 1943
Stanford University, September 22, 1943
University of California, November 24, 1943
Western Regional Laboratory, January 22, 1944
Stanford University, March 25, 1944

Peiping, China

Chairman: A. B. Fortuyn Secretary: F. T. Chu Members: 28

Rocky Mountain

Chairman: F. X. Gassner Secretary: A. G. Wedum Members: 26
Meetings: Denver, Colo., December 10, 1943
February 25, 1943
May 26, 1944

Southern

Chairman: K. L. Burdon Secretary: G. E. Burch Members: 42
Meetings: Tulane University, October 21, 1943
November 29, 1943

Southern California

Chairman: A. P. Hoyt Secretary: A. M. Schechtman Members: 48
Meetings: University of Southern California, October 12, 1943
California Institute of Technology, January 14, 1944
University of California, Los Angeles, May 29, 1944

Western New York

Chairman: R. K. Brewer Secretary: A. H. Hegnauer Members: 68

Wisconsin

Chairman: A. L. Tatum Secretary: L. E. Casida Members: 47

MEMBERSHIP

Members, March 31, 1943	1710	
Elected during year	96	
		1806
Resignations	14	
Arrears	6	
Deaths	12	
		32
Total Membership, March 31, 1944		1774

Membership:	1934	1944
	1218	1774

Subscriptions, March 31, 1944 1047

AUDIT REPORT

April 1, 1943-March 31, 1944

Statement of Income and Disbursements

INCOME

Dues		$ 6,644.86	
Subscriptions	$8,145.25		
Reprints	2,346.49		
Space	1,091.62		
Cuts	567.62		
Changes	27.97		
Sale of Back Numbers	693.95		
		12,872.90	
Interest—Federal Savings and Loan		1.99	
Miscellaneous		24.92	
Total Income			$19,544.67

DISBURSEMENTS

Office Supplies, Telephone and Postage		$ 892.16	
Cost of Printing	$7,754.32		
Cost of Reprints	2,359.67		
Cost of Cuts	768.90		
Refunds	89.10		
Storage and Insurance	74.75		
Purchase of Back Numbers	5.75		
		11,052.49	
Salaries		2,744.20	
Miscellaneous		81.24	
Total Disbursements			$14,770.09
Excess of Income Over Disbursements			$ 4,774.58

STATEMENT OF ASSETS AND LIABILITIES

AS OF MARCH 31, 1944

(Includes Surplus Fund)

Assets

Cash in Banks	$10,982.96	
Investments—Surplus Fund	18,975.29	
Life Membership Fund	76.12	
Accounts Receivable	869.27	
Total Assets		$30,903.64

Liabilities

Accounts Payable	$ 26.32	
N. Y. Section	234.72	
Withholding Taxes	106.60	
Total Liabilities		367.64
Net Worth		$30,536.00

Endowment Fund

Guaranteed Mortgages	$8,744.01	
Bowery Savings Bank	1,280.89	
U. S. Savings Bonds	7,550.00	
Industrial Bonds	2,923.62	
Total		$20,498.52

Pursuant to the request of your Board of Auditors, we have examined the books and records of the Society for Experimental Biology and Medicine for period April 1, 1943 to March 31, 1944.

We have checked the cash accounts in the Manufacturers Trust Company, Bowery Savings Bank, Harlem Savings Bank and the R. R. Federal Savings and Loan Association. We have also checked all your securities in the vault. We have found the accounts correct and well kept.

Respectfully submitted,
(Signed) Alexander Dolowitz, C.P.A.

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Carmichael, L. Tufts Coll.
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Cartland, G. F. Kalamazoo, Mich.
Carruthers, A. Birmingham, England
Cary, C. A. U. S. Dept. of Agriculture
Casals, Jorge Rockefeller Inst.
Casey, Albert E. Birmingham, Ala.
Casida, L. E. Univ. of Wisconsin
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Charipper, H. A. New York Univ.
Cheer, S. N. W. China Union Univ.
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Cope, O. M. N. Y. Med. Coll.
Copenhaver, W. M. Columbia Univ.
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